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EXPERIMENTAL EPIDEMIOLOGY.

INTRODUCTORY.

BY SIMON FLEXNER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 15, 1922.)

Each generation receives its particular impression of epidemic diseases; during the past 20 years the impressive epidemics of the western world have been those of meningitis or cerebrospinal fever, poliomyelitis, influenza, and latterly lethargic or epidemic encephalitis. These several epidemic diseases have numbered their cases by the hundreds, thousands, and even tens of thousands in given communities or countries, and have ravaged not only the United States, but within the same period have prevailed, sometimes with more, sometimes with less intensity, in other and distant parts of the world.

All these epidemics are indeed old-world diseases, and excepting the last, namely lethargic encephalitis, regarding which knowledge is still scant and uncertain, they can be traced far back in recorded human history. Moreover, the remarkably wide or pandemic outbreaks of which the recent American epidemics form part all originated in the old world and were communicated, usually after a lapse of time, to the new world, where, in some instances, as in the notable one of the 1916 wave of poliomyelitis, they found a soil so fertile and circumstances of spread so favorable as to reach a height of destructiveness previously unknown.

Ever since Hippocrates and especially since Sydenham, the study of epidemics of disease with a view to penetrating their hidden meaning has engaged the attention of occasional men. The degree of interest in what may be called the nature of epidemics has, however, fluctuated greatly and considerable periods have elapsed in which the subject has been given only superficial thought. Then

circumstances have arisen through which both the professional and the public mind has become so engrossed with it that new efforts come to be put forth in order to grasp its significance.

We are moving now in such a period of revived interest. Ever since the rise of modern bacteriology, the hope has been indulged that as knowledge of the microbic incitants of disease grew, the nature of epidemics would become more comprehensible. That this hope has not been entirely disappointed is shown by the influence which precise knowledge of certain disease-producing microorganisms, taken directly into the gastrointestinal tract or communicated by insect vectors, has had on the control of maladies thus induced. The discoveries in these two fields alone and the measures derived from them, as well as the discovery of the part played by those potential distributors of the microbes of disease called "carriers," have led to practical achievements of great magnitude in the domain of hygiene, imparting a sense of human power and control over impending epidemics of disease, in profound contrast with the helplessness of any previous period in history.

And yet in certain quarters, and especially in England, what may be defined as a reaction against the teachings of modern, that is bacteriological, epidemiology seems to have set in. That this return to older, and as it must seem, more mystical doctrines is widespread cannot be affirmed, but it has even happened that in the effort to elucidate the epidemic prevalences of the past two decades already enumerated and eventuating in the colossal outbreak of influenza, the notions of epidemic constitutions as defined by Hippocrates and especially by Sydenham have been more or less sported with, if not actually invoked.[1]

[1] Payne, J. F., Thomas Sydenham (Masters of medicine series), London, 1900. Greenwood, M., Sydenham as an epidemiologist, *Proc. Roy. Soc. Med.*, 1918–19, xii, Epidemiol. and state med. sect., 55. Goodall, E. W., Discussion on Sydenham, *Proc. Roy. Soc. Med.*, 1918-19, xii, Epidemiol. and state med. sect., 66. Singer, C., Discussion on Sydenham, *Proc. Roy. Soc. Med.*, 1918–19, xii, Epidemiol. and state med. sect., 71. Greenwood, M., The factors that determine the rise, spread and degree of severity of epidemic diseases, *XVII Internat. Cong. Med.*, 1913, sect. 18, 49. Report on the pandemic of influenza, 1918–19, *Ministry of Health, Rep. Pub. Health and Med. Subj., No. 4*, 1920.

To the extent to which this reaction is an endeavor to define epidemiology in terms wider than those of the microbic incitants of disease alone it may be regarded as wholesome and timely. The doctrine of "carriers" of potentially infecting microorganisms necessitates a conception of epidemic disease wider than is embraced in the exclusive view of their varying pathogenic activities. That the mere occurrence of potent microorganisms does not suffice to produce an outbreak of epidemic disease is a commonplace of bacteriological knowledge. The conditions are not as simple as that, and in their greater complexity they include not only the various qualities of the microbe but also of the host and, as well, their many reactions, one upon the other.

But it may be questioned whether progress is to be gained by a return to the indefinite concepts of epidemic constitutions or of the interdependence of diseases as various as epidemic meningitis, poliomyelitis, influenza, etc., united by an inevitable nexus of events the nature of which cannot even be surmised.[2]

Against these vague considerations modern bacteriology opposes the belief in a specific etiology, in which a particular microorganism is sought as the incitant of the infectious disease under study. The triumphs already secured in this field need not be recited here. But it is obvious that the gains made are not sufficient to account for all the phenomena of epidemics. The search, therefore, must, if possible, be both widened and deepened. Hence, the immediate question which presents itself is the manner in which this can be done.

[2] Hamer, W. H., The Milroy lectures on epidemic disease in England—the evidence of variability and of persistency of type, *Lancet*, 1906, ii, 569, 655, 733; Discussion on influenza, *Proc. Roy. Soc. Med.*, 1918–19, xii, Roy. Soc. Med. sect., 24; Discussion on Sydenham as epidemiologist, *Proc. Roy. Soc. Med.*, 1918–19, xii, Epidemiol. and state med. sect., 72. Newsholme, A., and Stevenson, T. H. C., et al., Discussion on influenza, *Proc. Roy. Soc. Med.*, 1918–19, xii, Roy. Soc. Med. sect., 1. Crookshank, F. G., First principles: and epidemiology, *Proc. Roy. Soc. Med.*, 1920, xiii, Epidemiol. and state med. sect., 159; A note on the history of epidemic encephalomyelitis, *Boston Med. and Surg. J.*, 1920, clxxxii, 34; Public health considerations relating to influenza, pneumonia, and allied epidemics. The epidemiological point of view, *Boston Med. and Surg. J.*, 1921, clxxxiv, 548. Report on the pandemic of influenza, 1918–19, *Ministry of Health, Rep. Pub. Health and Med. Subj., No. 4*, 1920.

Epidemic outbursts of disease occur among animals and pursue a course similar to that which has been observed to occur in man. The direct study of epidemics among animals under conditions of control not attainable in man should therefore commend itself to the epidemiologist. By this means it may be possible to secure those precise data of both microorganism and host on which eventually a real science of epidemiology may come to be built.

Thus an investigation was undertaken several years ago relating to certain aspects of experimental epidemiology of which the papers to follow are the first fruits. The papers relate to epidemics in mice of gastrointestinal origin, to which the name of mouse typhoid is applied. While mouse typhoid presents clinical and pathological characteristics of a single disease-complex, its microbic incitant is not a consistent species. Indeed, just as there are distinct but related bacilli inducing dysentery in man, there are distinct but related bacilli capable of provoking "typhoid" in mice. This relative multiplicity of microbic factors may be viewed as an aid rather than as a hindrance in investigating the events taking place in course of epidemics, the knowledge of which may come to have applicability to man as well as to the lower animals. The several bacilli alluded to differ little in cultural, but profoundly in immunologic properties. Some of the species exist either as saprophytes or at least as harmless "carried" bacteria in mice regarded as normal and yet become substituted for the original inciting microbes during epidemics—a fact of high importance in respect to the vagaries noted in the bacteriologic investigations of certain epidemics in man.

Not only do specific differences exist among so called mouse typhoid bacilli, but all the pathogenic varieties appear extremely labile. Strains of the bacilli artificially enhanced as they pass from mice to mice quickly fall to an average of infectivity and are, as it seems, at low pathogenic ebb at the time of the death of the infected animals. But this lability of the bacilli is determined, in part, by the hosts; that is, the mice through which they pass. In this respect mice may be viewed as consisting of different biological classes according as they respond to ingestion of the bacilli with infection and death, with mere carriage of the bacilli, or with non-reactibility. The distinctions of classes are not, however, absolute, but are determined, partly at

least, by the quantity or dosage of the bacilli. It is this latter factor which plays so conspicuous a rôle in the phenomenon of recurrent epidemic waves superinduced by the introduction of new mice in the replacement experiments described.[3] While it is the "carrier" among the old mice which provides the "seed" for the next following epidemic outbursts, it is the highly susceptible individuals among the new which furnish the living "culture" medium enabling rapid increase and wide dissemination of the bacilli to be effected, just as it is the succumbing and non-reactible mice which check the growth and multiplication that tend to arrest the epidemic spread.

These factors leave out of account the effect, if any, of active immunization acquired through previous exposure, a condition shown experimentally to be realizable, but the extent and sufficiency of which in initiating the events and finally in bringing about the state of equilibrium between parasite and host prevailing at the end of the epidemic wave are still undetermined.[4]

The papers in this series relate to a gastrointestinal infection in mice. In man the prevalent type of epidemic disease has altered notably within a century; there has been far less of the diseases in which the mode of infection is enteric and more of the diseases in which the portal of infection is by way of the respiratory tract, which fact leads to the consideration that the control which modern hygienic practises exercise over epidemics depends on the imposition of general or communal, as opposed to individualistic measures of prevention. Smallpox is controlled by essentially universal vaccination; typhoid and allied fevers by water purification and similar means; malaria, typhus, and yellow fever by war on their insect propagators. While conversely the epidemic diseases which still prevail almost unchecked among the western peoples are just those for which no communal means, practically applicable, exist of preventing communication of the potentially morbid materials, such as those of the secretions of the respiratory tract, from individual to individual. And coinci-

[3] Amoss, H. L., Experimental epidemiology. II. Effect of the addition of healthy mice to a population suffering from mouse typhoid, *J. Exp. Med.*, 1922, xxxvi, 45.

[4] Webster, L. T., Experiments on normal and immune mice with a bacillus of mouse typhoid, *J. Exp. Med.*, 1922, xxxvi, 71.

dentally with this circumstance, other significant conditions have arisen to modify previous history, namely the rise of multitudinous cities, industrialism, rapid transport, etc., the effects of which are to increase the number and intensity of personal contact associations, and thus to combine, confuse, and distribute quickly and widely the respiratory secretions of unnumbered persons.

It is our intention to extend the experimental investigation of epidemics among laboratory animals to the respiratory-borne infections. Opportunities for such studies undoubtedly exist. The obvious differences and inconsistencies between the animals thus affected and man, primarily in respect to the distinction in habits, may not prove insuperable obstacles in the way of obtaining illuminating information from the one applicable to the other. In any case it will be found desirable to check the experimental studies on epidemics of enteric origin with those of respiratory origin and to determine in how far they pursue similar and to what extent they follow different modes of evolution and devolution.

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EXPERIMENTAL EPIDEMIOLOGY.

I. AN ARTIFICIALLY INDUCED EPIDEMIC OF MOUSE TYPHOID.

BY HAROLD L. AMOSS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, February 9, 1922.)

The problem set ourselves was the study of an epidemic of mouse typhoid conducted under experimental conditions. The painstaking observations recorded in Dr. Lynch's paper,[1] far exceeding those usually accorded outbreaks of disease among domesticated animals, seemed to put rather than to answer questions of epidemiology. It appeared to us therefore that an epidemic started under fixed conditions, in which one person followed the happenings day by day and recorded the events, would not only tend to eliminate errors traceable to the elements of surprise and lack of preparation, but also by providing a more homogeneous material yield results of greater consistency, while the two major factors of host and parasite would be placed under highly favorable conditions of control.

Method.

Of laboratory mammals, mice are most easily assembled and observed in large numbers. Moreover, they are subject to a bacterial infection, mouse typhoid, of gastrointestinal origin, the pathology of which is quite well known. The lesions found in and characteristic of the disease affect several important viscera and are obvious to the unaided eye. The bacteriology also is sufficiently worked out so as to serve as a guide in what may be regarded as doubtful instances. Moreover, as is well known, the disease mouse typhoid constitutes a common sporadic fatal epidemic affection among mice and even from time to time sweeps through mice colonies in highly destructive waves.

[1] Lynch, C. J., *J. Exp. Med.*, 1922, xxxvi, 15.

In view of the experiences described in Dr. Lynch's paper,[1] our experimental investigations were started with mouse typhoid. While under way, Topley's[2] series of valuable papers on the same general topic began to appear in print. They will be discussed along with the deductions from our own observations in the proper place.

The procedure adopted by us at the outset was one chosen to simulate the conditions of epidemic outbreaks of disease not only among carefully segregated small domestic animals but also those which occur among human beings.

Thus what may be termed a mouse village was set up by placing in rows on metal shelves metal cages 7 by 10 by 5 inches with wire mesh tops. 5 mice were placed in each cage without communication between the cages, so that infection could be transferred only by the hands and implements of the person cleaning the cages and feeding the mice. Great care was taken throughout the experiment to exclude roaches, ants, flies, and other insects and vermin from the room. The temperature of the room was kept constantly at 68°F. whenever the outside temperature was below this. In the summer months the temperature of the room was, of course, higher. The cages were thoroughly cleaned once each week, always in the same order, and the mice were fed in the same order daily as follows: grain in the morning and bread moistened with milk in the afternoon. Precautions were taken to exclude extraneous disease. The mice used came from a carefully controlled healthy stock bred in the Institute and free of communicable disease.

There are certain advantages in keeping the experimental animals in small groups: it simplifies identification and stock taking, and the keeping of records; and allows frequent inspection for dead animals; it reduces the death rate resulting from fighting. The spread of infection is limited to one agent; *viz.*, the cleaning implements and hands of the caretaker.

Introduction of the Virus.—As already stated, when the study was begun Topley's papers had not appeared. His method of placing a known amount of culture on small bits of bread to be consumed by mice from which food had been withheld for 24 hours is efficacious

[2] Topley, W. W. C., *J. Hyg.*, 1920–21, xix, 350.

and more accurate than our first attempts by allowing hungry mice to drink milk from a tube. In order to give the exact dose to each mouse, we later introduced the bacteria suspended in milk directly into the stomach through a small silver tube. In this way the dose can be accurately measured and the use of more than 1 cage for each 5 mice obviated for the preliminary step of infecting the first mice.

Inspection.—The cages were examined three times daily except Sunday, when only one inspection was made. Even under these conditions it sometimes happens, especially on the first round of the day, that only a partially eaten body of a mouse is found. The survivors quickly attack the body of a dead mouse and devour the softer parts. Earlier in the experiments, when we had little experience, the number of deaths which could not be definitely determined as due to *Bacillus typhi murium* infection was proportionately larger than in the later series.

The bodies collected at 9 a.m. rounds were autopsied at 10 a.m. Those collected later were kept in the refrigerator (+4°C.) until the following morning.

Records.—In following the progress of the infection, two charts were kept: (*a*) plot of the total number of deaths for the whole series, according to days, and (*b*) spot map using colored pins showing the location of the mice which died and the number of days elapsed since the beginning of the exposure.

Autopsy Technique.—The notes at autopsy included the weight of the body and of the spleen, and a brief description of the macroscopic appearances of the spleen, intestines, liver, and gall bladder. The bacteriological examinations of the spleen, intestinal contents, and gall bladder were carried out in the following manner. A small bit of the material was transferred to a tube containing 5 cc. of brilliant green broth (brilliant green 1:200,000 in broth of pH 7.4) and crushed inside the tube. To the tube there was then added 0.25 cc. of a 1 per cent sterile aqueous solution of lead acetate. The tube was incubated at 37°C. over night. If on the following morning the precipitate in the tube was brown, showing evidence of sulfide production (presumptive test of the presence of *Bacillus typhi murium*), a loopful of the broth was smeared on lactose-saccharose-neutral red agar, pH 7.4, plates containing 1:400,000 brilliant green. Colonies from these

plates were fished into lead acetate agar tubes containing four sugars:[3] lactose, raffinose, saccharose, and salicin, 0.25 per cent of each. Cultures having the characteristic reactions of *Bacillus typhi murium* were then agglutinated with two monotypical immune sera. The longer incubation period for brilliant green broth tubes has revealed a higher percentage of deaths attributable to mouse typhoid than was obtained with the usual procedure of 1 hour's incubation in a water bath.[4] The addition of lead acetate to the broth greatly reduces the number of brilliant green plates required. The first few hundred examinations with this technique were controlled by the usual culture method resulting in a gain of about 10 per cent in the positive findings. The lead acetate did not fail in a single instance to reveal *Bacillus typhi murium*, and in 125 tubes, in which there was no browning controlled by plating, no *Bacillus typhi murium* was found. Many brilliant green tubes containing feces may show the sulfide reaction when no *Bacillus typhi murium* is present, due to the growth of other sulfide-producing organisms such as *Bacillus alkaligenes*, etc. The plating on brilliant green and transferring of the colonies to the four sugar tubes control this point.

The final agglutination test is highly important as endemic strains, differing antigenically from the strain used to induce the artificial infection, are sometimes found in mice succumbing in course of the epidemic experimentally set up.

[3] The composition of this medium is as follows:

Beef extract	3 gm.
Peptone	10 "
NaCl	5 "
Agar	15 "
Lactose	0.25 per cent.
Raffinose	0.25 " "
Saccharose	0.25 " "
Salicin	0.25 " "
Andrade indicator	1 " "
Distilled water	1,000 cc.

pH 7.2

Lead acetate ... 1 cc. of 0.25 per cent solution to each 4 cc. tube of medium.

[4] Undoubtedly in our earlier series many negative results were recorded which with the newer technique would have been found positive.

Bacillus typhi murium Employed.

A bacillus conforming to the cultural characteristics of *Bacillus typhi murium* and pathogenic for mice was isolated from an epizootic among a cancer breeding stock.[1] This strain (Mouse Typhoid I) was passed by intraperitoneal injection through 5 mice. A suspension containing 1:20 of an 18 hour culture on slant agar was then fed to a mouse, and on the death of this animal the bacillus recovered, after being identified culturally as belonging to the same group, was fed to 4 mice in succession. As it happened, later immunological tests showed the strain recovered from the final mouse of this series to be antigenically different from the original strain but having identical fermentative properties with those of so called *Bacillus typhi murium* and being pathogenic for mice. It is probable that this strain which we term Mouse Typhoid II was enzootic among the stock used in the preliminary experiments, replacing somewhere in the series of 4 mice the strain used originally to induce infection. It came out in later studies that the second large outbreak among the cancer breeding stock[1] was caused by a strain antigenically identical with this strain. However this may be, the strain acquired highly invasive powers, as the experiments to be described will show. From this point on, this strain appeared in the animals succumbing to the mouse typhoid arising in the course of our experiments except in a few instances in which an immunologically different strain was obtained in culture. The fact of this substitution of strains emphasizes the importance of making regular immunological tests on all strains employed in and recovered during the experiments.

In referring to this bacillus with which our experiments were conducted as *Bacillus typhi murium*, we would have it understood that it belongs to the class of bacilli embraced under this term and was later found to be indistinguishable by immunological tests from *Bacillus pestis caviæ* of the paratyphoid B group. The immunological reactions of the bacillus will be described in a separate communication.[5,6]

Preliminary Culture Feeding.

Feeding Series B.—November 2, 1919. 18 normal mice, after fasting 24 hours, were fed during 24 hours with milk containing 1:20 culture of Mouse Typhoid II

[5] Amoss, H. L., and Haselbauer, P. P., *J. Exp. Med.*, 1922, xxxvi, 107.

[6] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 97.

from an 18 hour growth on slant agar. During the next 18 days, 15 died and yielded positive cultures of the same bacillus in the feces and spleen; in 8 the bacillus was recovered also from the gall bladder. The deaths took place as follows: 3 occurred in 7 days; 4 in 8 days; 2 in 9 days; 2 in 10 days; 1 each in 11, 15, 17, and 18 days. On the 36th day the 3 surviving mice were killed, of which 2 proved fecal carriers. The bacillus was not found in the spleen or gall bladder.

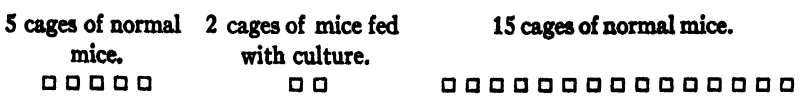
Series C.—The day after the feeding of the culture in milk just described, 13 mice (Series C) were placed in 2 cages of 5 and 8 respectively, and placed beside the cages containing the fed animals. They were all cared for by the same attendant. Of the cage of 5 mice, 1 only succumbed to mouse typhoid and that on the 12th day. Of the other cage, all 8 succumbed, on the 11th, 13th, 14th, 15th, 17th (2), 19th, and 25th days respectively. The unequal distribution of deaths in this series need not be considered now; but the main point is that already the culture Mouse Typhoid II had exhibited power to induce fatal infection through mediate contact as well as through direct feeding. Moreover, the contact mice succumbed in periods not exceeding those of certain of the fed mice.

Series D.—13 days after the feeding experiment of November 2, 24 mice in 8 cages (Series D) were placed beside the cages containing the 11 surviving mice of Series C. 11 died and 8 yielded positive cultures. At the expiration of 60 days, the remaining 13 were killed. 5 of them proved fecal carriers.

Experimental Epidemic.

The culture Mouse Typhoid II was now regarded as probably capable of producing mouse typhoid by ingestion in a large proportion of the fed mice and also of inducing that disease in exposed or contact mice not directly fed. Whether it possessed also the particular qualities which might be required in order that the spread from animal to animal should take place in the manner common in epidemics remained to be ascertained. An experiment to test this point was next designed.

Series E.—December 12, 1919. Food was withheld for 24 hours from 10 mice. Each mouse was then given milk to drink containing a heavy suspension of culture Mouse Typhoid II. It is estimated that each animal received approximately 1:20 of an 18 hour agar slant culture. At the end of 24 hours the mice were transferred to clean cages, 5 in each. With this experiment in view, 100 normal mice were assembled on December 8, into 20 cages, and arranged in positions indicated by the following diagram.



These mice had been free of fatalities. Just here it should be stated that the mice assembled for all the experiments were not only home-bred and of a stock free of disease, but when introduced into the experiment they were of an average weight of 13 gm. and age of 4 weeks.

The series of events were now as follows: Of the 5 mice in feeding cage 1, 2 died on the 8th, 1 each on the 13th, 14th, and 15th days. From all, cultures of Mouse Typhoid II were obtained. Of the 5 mice in feeding cage 2, 1 each died on the 12th, 13th, and 17th days. The 2 remaining mice were killed on the 60th day; cultures were negative.

Of the 100 mice in the 20 contact cages, the 1st animal died on the 15th day and the next on the 17th day. Within 60 days a total of 10 mice had succumbed. Of the 90 survivors, 7 (or 8 per cent) proved fecal carriers. Only 11 of the 20 cages showed infected mice as indicated either by death or carriage. The position of the mice, in respect to the purposely infected animals and the direction of the feeding or the cleaning of the cages, had no appreciable effect on the incidence of the infection. Obviously the attendant, in spite of more than ordinary precautions to keep his hands clean, early became contaminated and spread the contamination unwittingly and irregularly after the 1st day or two of his operations.

It is evident that what was produced in this experiment (Series E) was not an epidemic outbreak but rather a sporadic occurrence of mouse typhoid. But the impending conditions seem to have been significant. For on January 15, 1920, 79 normal mice in 17 cages (Series F) were assembled and placed on racks immediately next the cages of Series E on the 34th day after the beginning of that experiment. The effect was striking. No deaths occurred within the first 5 days, but there were 4 in the second 5 day period. The highest recorded were in the third 5 day period, and this wave quickly subsided. Two smaller waves appeared with their crests in the seventh and the ninth 5 day periods. The distribution of deaths in cages and periods is shown below.

Distribution of Deaths in Series F.

Days.	Deaths in cages.	Total No. of deaths in period.
1–4	0	0
5–9	Nos. 6, 13, 13, 16	4
10–14	" 1, 3, 4, 4, 5, 6, 7, 8, 9, 15	10
15–19	" 1, 2, 2, 4, 5, 6, 6, 10, 13	9
20–24	" 1, 4, 8, 11, 11	5
25–29	" 2, 3	2
30–34	" 2, 3, 11, 12, 15, 16, 17	7
35–39	" 2, 4, 13, 16	4
40–44	" 1, 8, 8, 8, 15	5
45–49	" 7, 13, 17	3
50–54	" 12, 14	2
55–65	" 13, 14	2

About 75 days after exposure the survivors of Series F, 24 in number, were killed and examined as carriers. 1 only was found. The following tabulation summarizes the results.

Total mortality	55, or	70 per cent.
Corrected "	41, "	52 " "
Cage attack rate	17, "	100 " "
Carriers among survivors	(1 in 24)	4 " "

A word of explanation is required regarding the terms total and corrected mortality. The latter refers to dead mice from which bacillus Mouse Typhoid II was recovered in cultures. The technique employed at this date was one in which the brilliant broth tubes were incubated for 1 hour before plating. Further studies showed that with this method many of these organisms are missed. This fact taken together with results obtained at a later date leads us to infer that the gross mortality figure as given is very nearly correct.

Thus it appears that once what may be termed sporadic cases of mouse typhoid are made to arise in a population previously free of this infection, the introduction of fresh, previously unexposed individuals may suffice, after a certain delayed or incubation period of 5 to 10 days, to bring about a sharp outbreak of cases which may reach epidemic proportions. In this particular instance every cage was attacked and the death rate very high.

Series G.—On February 13, 1920, or 29 days after the preceding or F series was started and at a time when the deaths among the latter had fallen, for the 5 days preceding, to 4, 48 normal gray tame mice of the average ages and sizes to the white mice employed up to this time, contained in 10 cages, were introduced into the mouse village and placed next the survivors of Series F. Instances of mouse typhoid arose among them and 25 mice died. On April 12, or 2 months after the placing of these mice in the village, the survivors were killed and examined for carriage of the bacillus Mouse Typhoid II. The following tabulation summarizes the results of this test.

Total mortality	25, or	52 per cent.
Corrected "	24, "	50 " "
Cage attack rate	10, "	100 " "
Carriers among survivors	(4 in 23)	17 " "

In brief, this series behaved very much as did the preceding one in respect to the several points covered in the study. It may be desirable

to point out the close correspondence between the total and corrected mortalities resulting from the improved bacteriological technique described on page 9.

It now became evident that it was possible to inaugurate epidemics of mouse typhoid among a healthy previously unexposed mouse population. Up to this time the events described are remarkably consistent. But as the experiment was continued by the successive introduction of fresh, vulnerable material, variations (or better stated perhaps wider fluctuations) made themselves apparent as we will now proceed to show.

At this point one or two incidental observations are called for. In the mouse room as arranged the racks carrying the cages were placed along the sides of two opposite walls, so that the cages could be made contiguous or could be separated by the distance of the width (12 feet) of the room. This separation did not in itself affect the closeness of contact, since in no instance did the mice actually mingle, but, as already stated, the intermediation of infected and non-infected animals was secured through the hands of the attendant.

However, the separation of cages by the width of the room made possible the carrying out of devices which might affect the mediation. For example, the attendant was made to use approved methods of hand sterilization[7] and to clean the cages and feed the mice on the side of the room away from the series in which the infection existed, before tending the latter.

Series H.—Thus on February 13, 1920, or the same day that Series G was brought into the village and placed in cages immediately adjoining those of Series F, 100 healthy mice were assembled in 20 cages (Series H) and placed by themselves on shelves along the opposite wall at a distance from those already in the room, where they were kept for 2 weeks. As deaths among these mice began within 2 weeks, it is evident that the carrying out of sterilization by the attendant did not suffice to render his hands free of contamination with the strain of bacillus employed in the infection experiments. The series was now brought over and placed next to Series I, described below. Deaths among them continued so that at the end of 2 months, April 12, the results were as follows:

[7] The cleaning of the hands of the attendant was carried out in the following order. 10 minutes scrubbing with hand brush, using tincture of green soap. Wash in warm tap water. 2 minutes in potassium permanganate solution. Then immerse in saturated solution of oxalic acid until stain is removed (3 to 5 minutes). Rinse in tap water.

Total mortality	33 per cent.
Corrected "	25 " "
Cage attack rate	(14 in 20) 70 " "
Carriers among survivors	(8 " 67) 12 " "

The lowered death rate and the reduced cage attack rate are at once apparent. That the events summarized in the tabulation accompanying Series H are not mere exceptions is shown by the next experiment.

Series I.—February 18, 1920. 177 healthy mice, distributed in 36 cages, were brought into the village and placed adjoining and in line with Series G. These healthy animals also were tended without sterilization of hands and tools, after Series G, in which the infection was still proceeding. The detached Series H was cared for first of all. It was supposed that should the infection appear among the mice of Series I, it would attain a degree of activity capable of being accelerated by the available mass of new infectible material introduced at about the period of the preliminary outburst, while the mice in the detached Series H might acquire some resistance due to slow infiltration of virus. As a matter of fact, the two series, H and I, behaved quite as if they had been one series and all the mice had been brought into the village at one time. The experiment involving Series I was terminated on May 18, or after 3 months. The tabulation gives the final outcome.

Total mortality	64, or 36 per cent.
Corrected "	47, " 26 " "
Cage attack rate	(28 in 36) 80 " "
Carriers among survivors	(14 " 113) 12 " "

Review.

It is not our intention to enter into a minute discussion in this place of the significance of these experimental data. Such discussion as we purpose to give the subject will be presented in connection with the next paper in which the succession of events taking place day by day in the several series will be described.[8] We prefer merely to pass in review in this place the salient facts connected with the experiments detailed.

The feeding of the mice exposed in the village and the cleaning of the cages were done by one person and always in the same direction.

[8] Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 45.

As each new series was introduced, its cages were first tended. Series G and I were brought into the mouse village when the epidemic in Series F was at the crest of the wave. Series F (79 mice) was assembled and exposed on January 15, Series G (48 mice) on February 13, Series H (100 mice at distance until February 27) on February 13, Series I (177 mice) on February 18. Series F, G, and I were contiguous; Series H was separated by the distance (width) of the room (12 feet). Before the cages of Series H were touched, the attendant scrubbed his hands and nails with soap and brush for 10 minutes and used permanganate of potash and oxalic acid as disinfecting solutions. After 2 weeks separation Series H was brought into contact with Series I.

The several series may be regarded as having been placed in a single line, with the cleaning and feeding carried out in one direction. Thus following Series F there are 10 cages of Series G containing 5 mice each, followed by 36 cages of Series I and 20 cages of Series H, also of 5 mice each. Numbering the cages in order, Series G would be covered by cages 1 to 10; Series I by cages 11 to 46; and Series H by cages 47 to 66 inclusive. It might be expected that mice would be first attacked and die in cages 1 to 5 and that the infection would spread by contiguity. But the order is not so regular as this and the contrary may happen, for the first mice to die of mouse typhoid were in cage 7; then in cages 2, 3, and 4; then in Nos. 2, 3, and 5; then in Nos. 9 and 10. In Series I, of 36 cages, exposed 5 days after Series G, the distribution was as follows:

Days.	Deaths in cages.
1–4	Nos. 20, 29
5–9	" 11, 16, 17, 23, 25, 34
10–14	" 18, 24, 27, 28, 33, 34, 39
15–19	" 11, 13, 14
20–24	" 13, 23, 30, 32, 34
25–29	" 13, 26, 32, 33
30–34	No. 30
35–39	Nos. 26, 27
40–44	" 24, 29
45–59	" 11, 19, 21, 24

The one safe deduction from this tabulation seems to be that a wide but not uniform distribution of the bacillus is quickly brought about by the attendant through which individual mice, in an entirely unpredictable order, take it up and fall victims to the infection ensuing. Once this has happened the cages must soon become contaminated widely by the excrement carrying the bacillus and all the remaining mice should receive the organism. The sources of the subsequent irregular events can only be inferred as depending upon such factors as dosage and possibly fluctuations in pathogenic activity of the bacillus, and upon variations in the resisting powers of the mice. These factors are the ones commonly invoked to explain such vagaries of case incidence of the communicable diseases as here presented and are set down here not as finalities or even as matters for discussion, but rather to emphasize a parallelism existing between the natural, so called, infections in man and animals and those purposely set up, as in the instance being considered, which may be used in putting the next question to be answered by experiment.

In concluding this presentation, a table has been prepared of the five series of mice exposed to infection with the bacillus of mouse typhoid (Table I). The number of mice in each series varied from 48 to 177. In the table, Series I, which contained the largest number given, is broken up into three parts called first, middle, and last, according to the degree of removal of the cages at the time they were brought into the mouse village from the mice which were already potentially infected. It is not suggested that the factor of nearness or remoteness is a controlling one, but the division is interesting as bringing out again the element in the process of distribution of the infection which for the present is merely termed vagary. It is to be kept in mind that the quality of mice in the three divisions was superficially homogeneous. The only mice fed with culture were 10 of Series E. The rest of the animals received the bacillus through the exigencies of contact between them, their habitation, and the hands of the attendant.

The figures in Table I not only speak for themselves but have already been discussed in the earlier pages of this paper. The exception is Series I in which the three groups of approximately 60 mice each, selected by degree of removal from the older infected series into

relation with which they were brought, show a mortality of 38, 56, and 15 per cent respectively. Since the first group of 60, immediately next the older and already infected series, gave a lower mortality than the middle and further removed group, the very low mortality in the last group can hardly be accounted for by position alone.

TABLE I.

Composite Table of the Five Series.

Series.	No. of mice in series.	Mortality.	Corrected mortality.	Carrier rate among survivors.	Carrier rate in total No.	Cage attack rate.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
E*	100	10	10	8	7	55
F	85	70	52	4	1	100
G	48	52	50	17	8	100
I First	60	38	22	11	7	83
I Middle	60	56	52	4	2	100
I Last	57	15	5	18	15	50
H	100	33	25	15	10	70

* The infection in this series was produced by feeding with a suspension of *B. typhi murium* 10 of the mice.

The tabulation brings out a striking irregularity between carrier and death rate. Thus of 14 carriers, 8 were found in the same number (8) of cages in which no deaths from mouse typhoid took place, while 6 were found in 29 cages in which deaths from the infection took place. The distribution of carriers according to deaths in the cages is as follows:

No. of deaths in cage.	Instances of carriage of bacillus.
0	8
1	3
2	1
3	2
4	0

Experiments with Strains Arising from a Single Bacillus.

Six strains were obtained from single cells of Mouse Typhoid II by Barber's method. In order to be certain that only single cells

were picked, the strains obtained were plated, a colony was picked, and from the 8 hour growth in broth a single cell was again isolated and the same process repeated. Thus before a strain was considered as arising from a single cell, the culture was analyzed by the Barber method three times.

Six such pure-line strains were obtained, three of which showed slightly greater virulence by intraperitoneal injection into mice, as shown in Table II.

TABLE II.

Results of Intraperitoneal Injection of 1:30,000 of a Culture of Each of Six Single Cell Strains of Mouse Typhoid II.*

Weight of mice.	Length of life. Strain.					
	A	D	E	F	G	H
gm.	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*
13	52	44	66	20	66	64
	60	60	78	54	72	66
14	75	78	100	64	84	72
	88	78	105	66	90	78
15	80	82	115	72	94	100
	90	86	134	78	134	138
16	96	94	150	84	150	200
	111	110	160	99	200	255
17	130	130	255	115	408	260
	140	132	S.†	200	520	495

* The suspensions were standardized in a turbidimeter.

† S. indicates survived.

Comparison of the Virulence of Single Strain A with the Composite or Original Strain of Mouse Typhoid II.—Since the results of intraperitoneal injections (virulence tests) of mice with the mouse typhoid organisms vary considerably, it becomes necessary to use large numbers of mice in each experiment. With the view of determining what mice grouped according to weight would yield the most concordant results, the following experiment was made.

Experiment 1.—Mice were selected from a large stock until 10 of each weight from 10 to 17 gm. were obtained. Three lots of 6 each, composed of individuals weighing 18, 19, and 20, and 1 weighing 21 gm., were selected. Each of the 99 mice received intraperitoneally in 1 cc. of salt solution 1:30,000 of a 16 hour slant agar culture of Mouse Typhoid II. This strain had been plated repeatedly so that the culture represents the descendants of a single colony.

The number of hours before death is recorded for each mouse in Table III.

TABLE III.

Relation of Weight to Susceptibility to Intraperitoneal Injection of Mouse Typhoid, Strain II.

November 30, 1920. Each mouse received intraperitoneally in 1 cc. 1:30,000 of a 16 hour growth on pH 7.4 agar; suspension standardized in a turbidimeter.

Length of life.											
Weight of mice.											
10–10.5 gm.	11–11.5 gm.	12–12.5 gm.	13–13.5 gm.	14–14.5 gm.	15–15.5 gm.	16–16.5 gm.	17–17.5 gm.	18–18.5 gm.	19–19.5 gm.	20–20.5 gm.	21–21.5 gm.
No. of mice.											
10	10	10	10	10	10	10	10	6	6	6	1
hrs.	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*
20	20	20	20	49	20	20	20	46	20	20	89
20	20	27	25	66	30	59	20	73	60	20	
25	20	28	40	72	52	64	20	86	70	30	
48	41	60	50	75	52	86	27	106	75	60	
50	63	60	65	78	60	89	30	115	91	67	
69	67	72	74	79	73	90	34	130	96	75	
86	67	83	75	86	90	118	75				
98	67	86	89	115	115	118	86				
106	86	93	93	125	130	118	88				
110	113	104	106	160	144	160	90				

The number of deaths recorded in each group at 50, 75, 100, and 150 hours shows little correspondence, so that no conclusion can be reached in the matter of the selection of the most suitable group for testing virulence. If, however, a single cell strain of this micro-organism is employed, there appears in the same kind of experiment a definite relation between the susceptibility and age groups, as is shown in Experiment 2.

Experiment 2.—120 mice were selected so that there were groups of 10 of each weight from 10 to 21 gm. Such mice received intraperitoneally in 1 cc. 1:30,000 of a 16 hour slant agar growth of single cell strain Mouse Typhoid II A. The suspensions were standardized in a turbidimeter to the density of the suspension used in Experiment 1. The results are shown in Table IV.

TABLE IV.

Relation of Weight to Susceptibility to Intraperitoneal Injection of Single Cell Strain Mouse Typhoid II A.

January 13, 1921. Each mouse received intraperitoneally in 1 cc. 1:30,000 of a 16 hour growth on pH 7.4 agar; suspension standardized in a turbidimeter.

Length of life.											
Weight of mice.											
10–10.5 gm.	11–11.5 gm.	12–12.5 gm.	13–13.5 gm.	14–14.5 gm.	15–15.5 gm.	16–16.5 gm.	17–17.5 gm.	18–18.5 gm.	19–19.5 gm.	20–20.5 gm.	21–21.5 gm.
No. of mice.											
10	10	10	10	10	10	10	10	10	10	10	10
hrs.	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*
50	20	26	14	20	20	40	70	16	18	96	90
58	20	28	16	60	58	58	86	75	90	124	117
64	22	58	52	67	60	64	88	90	96	136	168
66	26	68	70	70	74	70	90	106	132	150	168
70	34	70	74	85	75	80	111	117	134	188	186
74	42	74	106	100	88	90	132	142	158	202	206
85	52	90	114	132	148	124	130	184	266	248	208
90	100	96	122	132	168	140	140	230	304	260	260
154	108	108	140	150	254	312	250	264	308	296	404
158	115	115	180	163	256	S.	266	S.	624	404	630

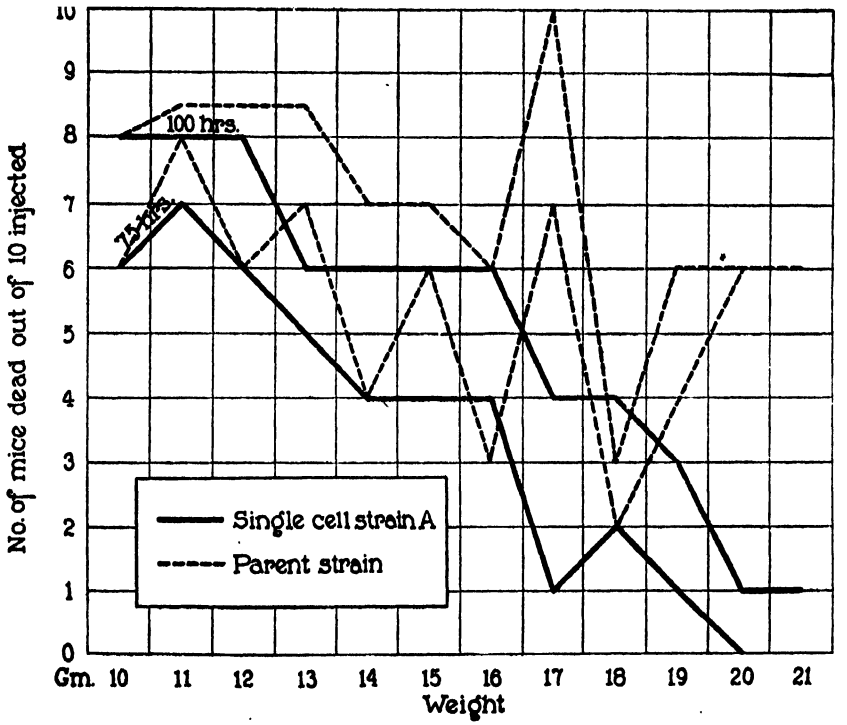
A comparison of the isolethal lines at 75 and 100 hours in Experiments 1 and 2 is shown graphically in Text-Fig. 1.

Of the two, the parent strain seems to be more virulent. The irregularity of the results in Experiment 1 and the obvious correlation between body weight and susceptibility shown in Experiment 2 with the single cell strain point to individual variation among the microorganisms comprising the parent strain.

Pathogenicity of Single Cell Strains Administered by Mouth. Experiment 3.—The power to infect by mouth of three of the single cell strains was tested by allowing groups of 8 mice each to drink milk containing living cultures of the strain to be

tested. Of 8 mice receiving Strain A, 5 died and 3 were living after 30 days; with Strain F 6 mice died and with Strain H only 2 died. Strain F was therefore selected in the experiment to be described, in which attempts were made to start an epidemic of mouse typhoid under the conditions known to be favorable.

Series O. Experiment 4.—February 10, 1921. Each of 10 normal mice from which food had been withheld for 24 hours was fed by stomach tube 1:250 of a 16 hour slant agar growth of pure-line Strain F.



TEXT-FIG. 1. Comparison of the relation of the virulence of a single cell strain and of the parent strain to body weight of mice. The injections were made intraperitoneally. The difference in weight of the 10 mice in a given group was not more than 0.5 gm.

February 11. The 10 mice in 2 cages, 5 in each, were placed midway on a shelf among 20 other cages, each containing 5 normal mice. During the following 46 days, 6 of the feeders and 7 of the contacts died of mouse typhoid.

On the 46th day of observation 100 normal mice in 20 cages (Series P) were brought into line with Series O. There was no sharp increase in deaths among the normal mice added or among the original. Thus among the new mice (Series P) the first death from mouse typhoid occurred 3 days later, and one on the 14th,

18th, and 36th days. Within 98 days only 11 of the mice died. During this period 10 of Series O died of mouse typhoid, making a total of 17 of the contacts in Series O and 6 of the feeders.

Since the addition of fresh normal mice in the above experiment with pure-line Strain F did not result in an outbreak of mouse typhoid under conditions which in our experience constantly incite epidemics with Mouse Typhoid II, another pure-line strain was employed in the next experiment under slightly different conditions; *viz.*, the new mice were added on the 31st day as in Series K, L, and M[9] instead of the 46th day as in Series O and P.

Series Q. Experiment 5.—June 8, 1921. Each of 10 normal mice from which food had been withheld for 24 hours was allowed to drink milk containing 1:20 of a 16 hour agar slant growth of pure-line Strain A, isolated from Mouse Typhoid II and belonging to the three more virulent pure-line strains.

On the following day the mice were placed in 2 cages, 5 in each, situated midway in a line of 20 cages, each containing 5 normal mice. During the following 30 days only 2 of the contact mice died. None of the feeders succumbed. On the 31st day, 100 normal mice in 20 cages (Series R) were added in line with Series Q. During the next 112 days only 7 of these mice died.

Comment.—Pure-line Strains F and A came from single cells picked from Mouse Typhoid II, the strain which was used to start the long replacement series, K, L, and M. It will be seen in another paper in this issue[8] that in the latter series the addition of new normal mice during a quiescent period was followed shortly by a new outbreak, first among the new or added mice and then among the old mice; that is, mice which had been for some time exposed to the virus. Under these same conditions of feeding, arrangement, and time under which the epidemic waves in Series K, L, and M were established with Mouse Typhoid II, the pure-line strains signally failed to induce an epidemic. Thus the individuals from which Strains F and A descended lacked the power to produce an epidemic under the same conditions which sufficed for the composite strain Mouse Typhoid II to incite an epidemic outbreak.

[9] These series are described at length on p. 28.

SUMMARY.

In this paper we have described the first part of an experimental study of the epidemiology of mouse typhoid. One set of data has been presented on the basis of which little or no analysis has been attempted. The immediate object has been rather to collect materials than to undertake to account for the phenomena encountered. It is obvious that the factors involved in the inquiry are intricate, but it is believed that they are not necessarily or all beyond disentanglement. About 500 mice in all have been studied in an experimental village, brought together in increments among a population in which mouse typhoid experimentally induced was prevailing.

The results have been presented according to two phenomena; namely, mortality or death rate, and bacillus carriage rate. The material does not lend itself to consideration according to morbidity rates. If it were established that every instance of attack, when not fatal, was attended by carrier production for the bacillus of mouse typhoid, reliable morbidity tables could be constructed. In the absence of this certain criterion, the materials here presented can be dealt with only as mortality data. This fact is attended with obvious disadvantages in respect to the epidemiological material assembled regarding infectious disease in man. In spite, however, of the drawbacks, it is already evident that the results obtained by the sort of inquiry here described may come to throw no inconsiderable light on moot problems on the origin, mode of spread, and manner of decline of epidemic diseases in general.

The analysis of the strains by selecting single cells and thus establishing substrains has yielded results which may eventually have value in explaining fluctuations in virulence. Among the positive data arising from the experiments with such cultures are, first, that there have been obtained by mechanical means from the ordinary bacteriologically pure culture, single cell strains exhibiting slightly different pathogenic activity, whether administered by mouth or parenterally, and second, that more regular results are obtained with intraperitoneal injections of these strains than with the parent strain. Among the negative results to be recorded are the failures of two single cell strains to incite an epidemic among mice under conditions known to be suitable when the parent strain is used.

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EXPERIMENTAL EPIDEMIOLOGY.

II. EFFECT OF THE ADDITION OF HEALTHY MICE TO A POPULATION SUFFERING FROM MOUSE TYPHOID.

BY HAROLD L. AMOSS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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In earlier papers of this series an ordinary or spontaneous epidemic[1] of mouse typhoid and an artificially induced outbreak[2] of the same disease have been described. The purpose of the present paper[3] is to consider more minutely the effect of bringing a healthy stock of mice into a community in which mouse typhoid is prevailing. The essential fact that such an addition of healthy mice leads to the transfer of the infection from the old population to the new comers, and the gross mortality figures thus resulting[2] have been pointed out. But the manner of the spread of the disease from the old to the new, and the reaction or effect of the revived epidemic on the old and previously surviving individuals, need to be dealt with in detail.

Method.

Three series of 100 mice each were assembled separately. The first, Series K, was used to start the outbreak by placing in it 10 mice which had been fed with living cultures, while the other two, Series L and M, were isolated to be brought 30 days later into contiguity with the first. After the three series had been brought together the infection spread, resulting in an outbreak which then subsided to a low ebb in which no deaths occurred for 1 week. Other new healthy mice were then introduced to bring the total number up to the original strength.

[1] Lynch, C. J., *J. Exp. Med.*, 1922, xxxvi, 15.

[2] Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25.

[3] An abstract of this paper was presented at the 36th Session of the Association of American Physicians, Atlantic City, May, 1921 (Flexner, S., and Amoss, H. L., *Tr. Assn. Am. Phys.*, 1921, xxxvi, 34).

After the new wave thus excited had receded, it became the rule to recruit again, by the introduction of healthy mice. Twelve such replacements were made during the period covered by this report.

EXPERIMENTAL.

Exciting the Epidemic.

Experiment 1.—Series K: May 1, 1920. 100 healthy mice were assembled in 20 cages of 5 each. May 3. 1 mouse found dead; no *B. typhi murium* detected. May 13. Second mouse found dead and *B. typhi murium* isolated from feces.[4] May 20. As no further deaths occurred, there were placed, midway in the series, 2 cages of 5 mice each, which had been fed during 24 hours on milk containing a suspension of *B. typhi murium*. It is estimated that each of the 10 mice received about 1:20 of an 18 hour agar slant culture. During the next 23 days 12 deaths occurred from mouse typhoid, 6 among the 10 fed and 6 among the 100 contact mice. The last of these deaths occurred on June 21, on which date Series L and M were brought into the room containing the infected Series K.

Series L: 100 mice had been assembled also on May 1. Between this date and June 21, 3 of the mice had died, and *B. typhi murium*[5] had been recovered from the spleen and feces in each instance.[4] On June 21 the 20 cages of Series L were placed in a row immediately adjoining those of Series K.

Series M: This series,[6] numbering 100 mice, had also been assembled on May 1. Between that date and June 21, when the 20 cages were brought into the room with infected Series K and placed immediately next the cages of Series L, 3 mice had died, and *B. typhi murium*[5] had been obtained from the spleen and feces of each.[4]

It is shown in Text-fig. 1 that the fatalities in Series K resulting from contact with the fed mice were very low—6 per cent. With-

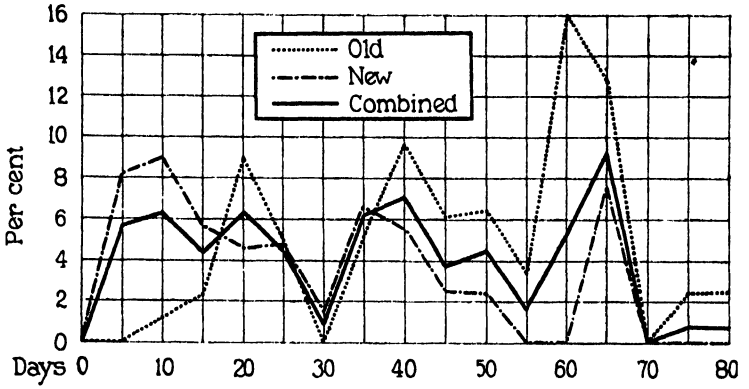
[4] Cultures from the spleen and gall bladder were negative. Three deaths from mouse typhoid occurred in each of the other two series, L and M, before exposure to the experimental infection. All came from the same stock of about 3,000 mice among which there occurred 6 deaths from mouse typhoid during the spring of 1920. It is presumed that in assembling the series one or more carriers was included. However, the series were for this reason kept isolated for 20 to 30 days before the experiment was begun.

[5] At this time the differences in Mouse Typhoid I and Mouse Typhoid II were not known.

[6] Series L and M were identical. The former was contiguous with Series K and the latter 20 cages removed from Series K, but contiguous with Series L. These two series are kept separate in order to observe the effect of distance from the source of infection on the manner of spread, and also for convenience in keeping records.

in 5 days after Series L and M were brought in, 6 deaths occurred in Series L and 9 in Series M, a total of 15; and in the next 5 day period a total of 19 deaths occurred in the two series. No deaths occurred in Series K during the first 5 day period and only 1 death in the second 5 day period.

The immediate result, then, of the introduction of normal animals was an outbreak among those mice brought in, whereas deaths in Series K did not increase until during the third 5 day period. The epidemic progressed in a succession of waves in each of the three series. In the like Series L and M, the waves were synchronous throughout the period, and the crests were lower and the intervals between greater



TEXT-FIG. 1. Death rate by 5 day periods in Series K, combined death rate for Series L and M, and combined rate for the three series, during the first epidemic period.

as the epidemic proceeded. The wave in Series K lagged behind Series L and M, and in contrast rose higher and the intervals between them became shorter. The lag of Series K decreased until at the twelfth period the wave of this series was coincident with the smaller Series L and M waves.

The three series were kept intact and without increments for 70 days after the normal mice had been brought into contact with Series K, at which time the epidemic had reached a low ebb. The total mortality among the approximately 300 mice in the entire period of 85 days after the introduction of the new mice was 54 per cent; in Series K 62 per cent (56 out of 91); in Series L 40 per cent (38 out of 97); and in Series M 61 per cent (59 out of 97).

The mortality among the added Series L and M combined was 50 per cent, while for the same period, the mortality in Series K (exposed from the first to the culture-fed group) was 62 per cent.

Comment.

The conditions of the experiment involving Series K, L, and M were similar to those described in the first paper of this series, in which first a sporadic outbreak of mouse typhoid was started by bringing a small number of mice purposely fed with a culture of *Bacillus typhi murium* into an assemblage of healthy mice; and the sporadic incidence was later fanned into an epidemic outbreak by the introduction of fresh groups of healthy mice at a time at which the occasional deaths had practically ceased. Hence the events recorded in the former paper seem not to have been accidental in nature but rather to follow a certain rule.

The course of the fatalities in the K, L, and M series is a definite one. In the first place the distribution of deaths in the cages was such as to point, just as in the earlier experiments, to an early general dissemination of the bacillus. Thus the first fatalities occurred in cages remote from those of the fed mice. But more significant is the fact that once the infection appeared among the mice of the L and M series, the number of fatalities not only rose steadily to a maximum, but was reflected backward to the K series in which no further deaths were taking place at the time. The precise manner of the rise in the L and M series, the exact period of delay in the K series induced by the occurrences in the former series, and the final result, or sum total of fatalities in each series, are shown graphically in Text-fig. 1.

To undertake to account for these happenings is to enter, at the moment, the realm of speculation. Two possible explanations present themselves. According to one it may be supposed that the bacillus of mouse typhoid was whipped into an exalted virulence by being rapidly passed from one susceptible mouse to another until this was so great as not only to reach a degree of infectivity greatly augmented for the new mice of Series L and M, but also to increase its striking power for the mice of Series K which, for a 30 day period, had successfully weathered exposure to the purposely fed mice introduced into the midst of that group.

According to the other supposition the effects described do not depend primarily on augmentation of pathogenicity but rather on increase in mass of the bacillus and result not from virulence so much, or alone, as from dosage. This view involves the conception that favorable conditions of growth and multiplication of the bacilli in the exposed mice are the more decisive events. It would seem as if these favoring circumstances did not exist in the first series employed but did arise subsequently among the new exposures. Once, however, they were secured, the growth and multiplication became such as to overwhelm not only the new series but also the older, previously exposed, survivors, among which the death rate was finally greater than among the new.

Probably the precise differences in the percentage mortalities are not significant. Series L and M were practically homogeneous and yet the one (L) gave a mortality of 40, and the other (M) of 61 per cent, the latter agreeing almost exactly with that of the K series. Moreover, according to this comparison, there are no grounds for supposing that the survivors of Series K by merely having been previously exposed were better able to withstand the violence of a real epidemic than were the previously unexposed mice of Series L and M.

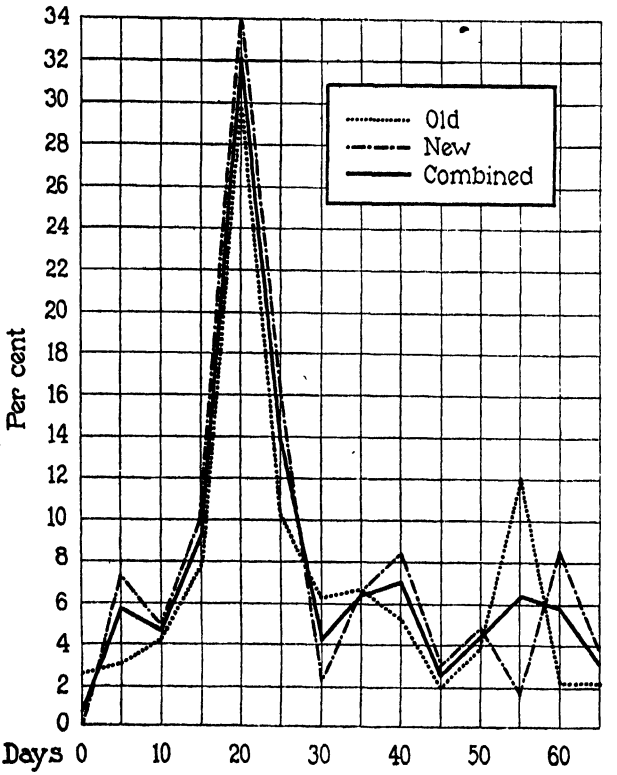
Hence it may be that the two factors mentioned, namely virulence and mass, are not only of high importance in themselves but also subject to such a degree of accurate measurement as to determine the potentially effective degree of each. All these considerations leave out of immediate account variation in the host—a factor at present not to be dealt with.

Effect of Replacements.

First Replacement, or R 1. Experiment 2.—September 9, 1920. The survivors of Series K, L, and M were observed during a period of 115 days at the expiration of which deaths took place seldom; during the final period of 7 days, no deaths at all occurred. At this juncture new mice from a healthy stock[7] were added to each cage in numbers replacing the deaths, so that each cage again contained 5 mice and the total population in the room was recruited to 300. In order to

[7] Among the stock of about 3,000 mice from which these mice were taken, there occurred 5 deaths from mouse typhoid in 6 months.

prevent losses from fighting between the old and the new mice, the latter were separated from the former by placing a coarse wire screen diagonally in the cage, separating it roughly into two compartments. While the mice themselves were thus grossly kept apart, yet they and their excrement came into contact.



TEXT-FIG. 2. Death rate by 5 day periods among the old and new mice, and combined rate, during the first replacement period.

The constitution of the several series as recruited was as follows: Series K now consisted of 98 mice,[8] of which 38 were old and 60 new; Series L of 100, of which 57 were old and 43 new; Series M of 100, of which 27 were old and 73 new. Hence the relation of old or exposed to new or unexposed mice totalled 122 : 176.

The new events came quickly. Within 5 days 17 deaths occurred, 13 among the new and 4 among the old mice. In the next 5 days

[8] This series was recruited to a total of 100 mice but 2 were killed in fighting.

there were 13 deaths, 8 among the new and 5 among the old. In the third 5 day period the deaths numbered 25, 16 of which were among the new and 9 among the old animals; while in the fourth 5 day period (20 days after the admixture of old and new mice) 79 deaths occurred, 48 of which were of new and 31 of old. This constituted the peak of the epidemic, after which the death rate fell off sharply.

These data are presented graphically in Text-fig. 2, from which it will be observed that the outbreak proceeded in two quite distinct waves among the new mice and in a steadily increasing progression among the old mice. This replacement, afterwards referred to as R 1, was observed for 65 days, during which a gross mortality from mouse typhoid of 69 per cent, made up of mortality K 86 per cent, L 68 per cent, and M 52 per cent, was noted.

Agglutinins in the Serum of the Survivors.

The serum of 56 of the survivors of the first replacement series was tested[9] for agglutinin with Mouse Typhoid II in dilution from 1:20 to 1:160. The serum from 37, or 66 per cent, partially agglutinated the strain in a dilution of 1:40, and of these, 20 showed complete agglutination in 1:40 and 2 showed complete agglutination in 1:160. The serum of 27 of the 56 mice showed no agglutinin in 1:20. Further analysis of the results shows that the serum of the 20 mice agglutinated the strain completely in a dilution of 1:40,

[9] The blood was collected from the mice by bleeding from the end of the tail. The mouse to be bled was placed in a small mailing case, one end of which was perforated and a single hole made in the other cap for the protrusion of the tail. The case containing the mouse was held in a clamp over a microscope lamp, the heat from which caused increased blood flow to the tail vessels. The tail was wiped with gauze moistened with 70 per cent alcohol, and after snipping with sharp scissors the drops of blood were allowed to flow by capillarity into a small bore, 1 cc. pipette graduated in hundredths. When 0.05 cc. had been collected, the end of the pipette was placed in isotonic salt solution and suction applied with a syringe until the mixture attained a total volume of 0.5 cc. The mixture now containing 10 per cent blood was delivered immediately into a small agglutination tube, mixed thoroughly, and allowed to clot. The following morning the clear fluid containing serum 1:20 was pipetted off with a small bore pipette, a syringe being used for suction.

while in 36 agglutination was either partial in 1:40 or absent. Of the former only 1 mouse, and of the latter 8 mice died during the second replacement series. Thus the death rate among the mice giving slight or no evidence of blood agglutinins was four times that noted among those possessing blood agglutinins.

Urinary Carriers.

Cultures of the urine of the same 56 mice were made on five occasions from November 15, 1920, to January 17, 1921. 35, or 62 per cent, were positive one or more times; of these 6 were positive during the entire period of 63 days. The serum of 5 of the 6 persistent urinary carriers agglutinated Mouse Typhoid II.

The results of the urine cultures and agglutination tests on the serum of the 56 mice are shown in Table I.

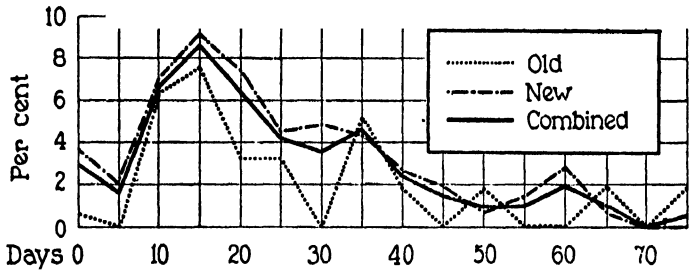
TABLE I.

Urine Cultures and Serum Agglutinins in 56 Survivors of the First Replacement Series.

	No.	Per cent.
Both agglutination and urine cultures positive	25	45
Agglutination positive, " " negative	12	21
No agglutination, urine cultures positive	10	18
" " " " negative	6	11

With these data before us we may now view the relative responses of the old or previously exposed and the new or not previously exposed mice to the impending infection. The number of old survivors which had already passed through an epidemic enduring 85 days and of which the total mortality was 58 per cent was 122. The death rate among these surviving animals induced by the new epidemic was 66 per cent, while the death rate among the 178 new or previously unexposed mice was 71 per cent. The percentage difference is too small to be significant, hence in all respects but one, namely that the new mice began to succumb before the first of the old, the two groups behaved in an identical manner. It is obvious that in the course of the second epidemic wave the gross mortality among the older mice exceeded that of the first epidemic, the proportions being as 58:71.

Second Replacement, or R 2. Experiment 3.—November 9, 1920. When the effects of the first replacement had subsided and no deaths at all occurred in a 5 day period, new mice, previously unexposed, were brought in as in the first replacement, to recruit the survivors in the three series to a total strength of 300. This second replacement was made 60 days after the first. The actual number of new mice introduced was 228, distributed as follows: Series K 87, Series L 82, Series M 59. Hence the proportion of old survivors to new population was as 72:228.



TEXT-FIG. 3. Death rate by 5 day periods among the old and new mice, and combined rate, during the second replacement period.

Text-fig. 3 shows that the new or R 2 mice began to die within 5 days, and 5 days later, or 10 days after the beginning of the experiment, deaths occurred among the survivors of the preceding epidemic. The experiment was allowed to run 75 days, at the expiration of which a total of 113 mice, or 38 per cent, had succumbed. The mortality in Series K was 29, L 40, and M 44 per cent, and among the old and the new mice it was 37.5 and 37.7 per cent, or virtual identity. That the old mice possessed no advantages over the new is further indicated by the patent fact that the number of the new (228) was approximately three times that of the old (72), thus increasing the probability of higher attack rate among the former. Ultimately the factors of age and size of the host may call for consideration. In those respects the surviving old differed materially from the younger and smaller new animals.

Third Replacement, or R 3. Experiment 4.—January 20, 1921. The third replacement of 114 new mice to recruit the new series up to 300 was made 75 days after the second. The series additions were almost equal: 66 in K, 65 in L, and 55 in M. During the next 60 days the total mortality was 14.3 per cent, distributed as regards old and new mice in proportion of 14 (old) to 14.9 (new) per cent.

This experiment was permitted to continue without interruption or modification for a period of 95 days, and it is worth while following the particular events which transpired. But first a few words are needed about the nature of the population composing the experiment. As is evident, the population is made up of mice of very different grades of exposure to mouse typhoid, since there have accumulated within it all the survivors of previous epidemics extending from May 20, 1920, of the first lot exposed, to January 20, 1921, when the third replacement was made. As Table II shows, the number of survivors of each replacement grows progressively smaller with each succeeding epidemic. Thus at the time of the third replacement period, what may be termed the combustible material consisted largely

TABLE II.

Distribution of Deaths According to Period of Introduction during the First 60 Days of the Third Replacement Period.

	Period of introduction of mice.			
	First epidemic (R).	First replacement (R 1).	Second replacement (R 2).	Third replacement (R 3).
No. of mice present	14	30	142	114
Deaths during first 60 days of Period R 3	1	2	21	17
Death rate, *per cent*	7	7	15	15
Survivors at 60th day of Period R 3	13	28	121	97

of the mice of the R 2 period. At the beginning of the R 3 period there were 14 mice remaining from the R period (first epidemic period), 30 from the R 1 period (first replacement), and 142 from the R 2 period. Reference to Table I shows that the mortality rate among the mice remaining from the R 2 period and the new mice was the same (15 per cent in each instance) and twice the rate observed (7 per cent) among the mice remaining from the R and R 1 periods.

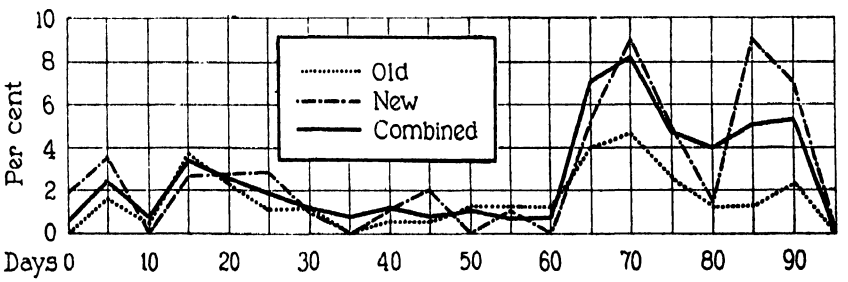
The events referred to may be summarized as follows: From the 25th to the 60th day, only an occasional death occurred in any of the series; for example, in Series K no death for 18 days; in Series L none for 29 days; in Series M none for 10 days. But at about the 60th day the deaths had reached epidemic porportions. This wave.

as it may be termed, was not induced intentionally but arose in the manner of similar waves in so called spontaneous epidemic outbreaks. Its duration was about 30 days, during which 73 (28 per cent) of 259 surviving mice after the first effect of the replacement subsided had succumbed. The distribution of deaths during this time, according to the period at which the mice were introduced, is given

TABLE III.

Distribution of Deaths According to Period of Introduction from the 60th to the 96th Days of the Third Replacement Period.

	Period of introduction of mice.			
	First epidemic (R).	First replacement (R 1).	Second replacement (R 2).	Third replacement (R 3).
No. of mice present at 60th day	13	28	121	97
Deaths from 60th to 96th days	0	4	35	34
Death rate, *per cent*	0	14	29	35
Survivors	13	24	86	63



TEXT-FIG. 4. Death rate by 5 day periods among the old and new mice, and combined rate, during the third replacement period.

in Table III. The death rate varied roughly inversely as the length of time the mice had been previously exposed. This higher mortality of 28 per cent is to be contrasted with the lower 14 per cent induced immediately by the repacement. The total mortality arising during the 96 days of the continuance of the experiment involved 114 of 300 mice, or 38 per cent. The death rate for each 5 day period is shown in Text-fig. 4.

Fourth to Tenth Replacements. Experiment 5.—From this point on small additions of new mice were made to the cages and the effects noted. They will not all be recorded in detail although the general effect of the new additions was deaths first among the new and then among the old. Table IV gives the size of each accretion and the mortality attached to each replacement group.

The death rate among the new mice was always in excess of that among the old. In short periods this is to be expected from the general rule that the new mice succumb first. In the table, mice surviving an exposure of 6 to 15 days are regarded thenceforth as old mice, whereas strictly speaking they are neither old nor new. Our impression is that mice exposed to an epidemic over 30 days are to be regarded as old.

TABLE IV.

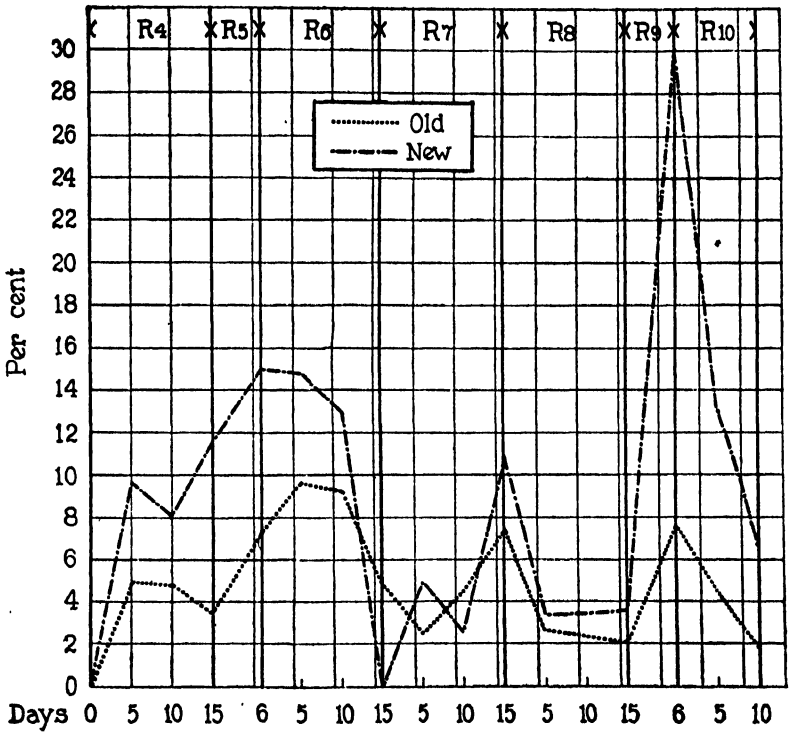
Deaths among Old and New Mice during the Fourth to Tenth Replacement Periods.

Period.	Duration of period.	Total No. of mice at beginning of period.	Old mice.	New mice.	Deaths during period.			
					Old mice.	Per cent.	New mice.	Per cent.
	days							
4	15	239	198	41	25	13	7	17
5	6	247	207	40	15	7	6	15
6	15	253	226	27	49	18	7	26
7	15	237	197	40	28	14	7	17
8	15	244	214	30	17	8	3	10
9	6	244	224	20	17	8	6	30
10	10	257	227	30	14	6	2	6

The general effect of the addition of the new mice is to maintain the death rate, as Topley has shown. The wave-like character of the fluctuations is evident from Text-fig. 5. The highest rates among the new mice are recorded in the sixth and ninth replacement periods; the interval between the beginning of the frequent replacement series and the sixth is 36 days, and between the sixth and the ninth is likewise 36 days.

Eleventh Replacement, or R 11. Experiment 6.—The effect of the addition of 30 new mice to the 227 old mice of the tenth replacement was negligible, as during 10 days only 1 mouse died; however, within the same time 13 of the old mice succumbed to mouse typhoid. At this point, July 15, 1921, what will be called the eleventh replacement was begun. At the time there were 62 cages in the mouse

village containing 183 mice. Each cage was recruited up to its normal content of 5 animals, thus giving a total population of 310 mice, of which 183 were old and 127 new. The death rate rose sharply among the new mice, and this was succeeded, after an interval of 5 days, by a rise among the old. The higher death rate among the new mice ceased at the 25th day, but later continued at about the same low level as that among the old during the succeeding 50 days (Text-fig. 6). The total mortality from mouse typhoid was 30 per cent, or among the new 31



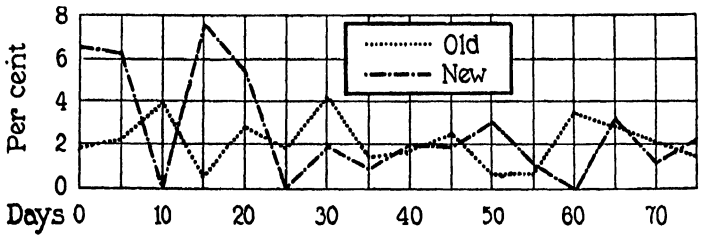
TEXT-FIG. 5. Death rate by 5 day periods among the old and new mice during the fourth to tenth replacement periods, inclusive.

per cent and the old 29 per cent, or as expressed in a proportion of old and new mice succumbing 52:40.

Twelfth Replacement, or R 12. Experiment 7.—October 18, 1921. The twelfth or final replacement was made in such a manner as to give the bacilli ample opportunity to infect new normal mice by retaining a small number of old mice and supplying five times as many new. All of the old mice in Series L and M, and all but 61 of Series K were discarded. To the latter there were added 54 new normal mice, bringing the total of the K series to 115. Series L and M were entirely

replaced by new mice, 100 in each. The three new series, K, L, and M, were now brought together in their usual order. The total number of mice was now 315, of which 254 were new and 61 old remaining from Series K. The ratio of old to new was about 1:4. The series was kept under observation for 60 days with the following results.

2 new mice were killed in fighting on the 1st day, reducing Series K to 113. The total number dying of mouse typhoid infection was 18. Mouse Typhoid II (the epidemic strain) was isolated from 12, and Mouse Typhoid I was isolated from 6. Of the latter, 3 were replacement or new mice.



TEXT-FIG. 6. Death rate by 5 day periods among the old and new mice during the eleventh replacement period.

TABLE V.

Deaths among Old and New Mice during the Eleventh and Twelfth Replacement Periods.

Period.	Duration of period.	Total No. of mice at beginning of period.	Old mice.	New mice	Deaths during period.			
					Old mice.	Per cent.	New mice.	Per cent.
	days							
11	75	310	183	127	52	29	40	31
12	60	313	61	252	12	20	24	9

The total mortality due to Mouse Typhoid II was therefore 12 out of 113, or 11 per cent, as follows: 9 deaths, or 15 per cent, among the 61 old mice, and 3, or 6 per cent, among the 52 new mice.

Among the 200 mice, all new, in Series L and M, the mortality was 10 and 7 per cent respectively. Mouse Typhoid II was isolated in each instance. The replacement and death figures of Periods 11 and 12 are shown in Table V.

The failure of the addition of new mice to bring about the usual response is doubtless to be explained by the fact that the bacilli were of low virulence.

The deaths due to Mouse Typhoid I call for some explanation. In the preceding series this strain was occasionally isolated, so that it was known to us that it had come in, probably, through stock mice. It is to be supposed that among the 61 old mice taken from Series K there were some carriers of Mouse Typhoid I, and that it was transferred to the new mice added. However, the infection did not spread to the new mice in Series L and M, as Mouse Typhoid II was isolated from each mouse dying in these two series.

The cultures of the spleens from 30 of the mice dying in the twelfth replacement were plated, and from each plate from ten to fifteen colonies were picked. Of the 400 cultures thus obtained, all belonged to Mouse Typhoid II as shown by agglutination with monovalent antiserum.

Mouse Typhoid Carriers.

On September 23, 1921, the total number of surviving mice of Series K, L, and M of the eleventh replacement period was 208, distributed as follows: Series K 79; Series L 70; Series M 69. To them calomel and castor oil were administered, mixed with food so that each mouse received approximately 4 mg. of calomel and 1 cc. of castor oil. The next day portions of the loose stools were easily collected on a platinum loop and cultured. Fourteen positive cultures of bacilli belonging to the Salmonella group were obtained. Of the colonies subcultured and tested for agglutination, only one reacted positively with the antiserum of Mouse Typhoid II. The remaining thirteen were not identified immunologically. This result is reported for two reasons, first, as again indicating the occurrence of members of the Salmonella group in the intestinal contents of so called normal mice, and second, that the detection of carriers of any particular Salmonella organism used for experiment cannot be based on cultural properties alone. Since only five colonies were picked from each plate, it is possible, of course, that the subculturing of many colonies from the stools of the mice found to be positive for *Bacillus typhi murium* might have yielded organisms agreeing immunologically with the strain employed in the experiments.

Immunity.

In a study conducted concurrently with certain of the observations presented in this paper Webster[10] found that mice which had been fed sublethal doses of cultures of *Bacillus typhi murium* were rendered resistant or immune to doses *per os* of the living culture fatal to control mice. This state of increased resistance depended not on local conditions in the intestine but on a general state of immunization attended by the appearance of agglutinins in the blood. It is well known that mice may be protected, at least partially, from parenteral inoculations of the bacillus of mouse typhoid by the injection of killed cultures, and in view of the general immunization thus induced and arising from the ingestion of the bacilli under conditions not leading to death from mouse typhoid, it was deemed desirable to test the survivors of certain of the replacement series with lethal doses of *Bacillus typhi murium* injected intraperitoneally.

Experiment 8.—September 29, 1921. The survivors of the various replacement series L and M, including all periods except R 3, were chosen for the purpose. They numbered in all 128 mice which had been exposed to mouse typhoid from 2 to 18 months before and continued to live in the presumably contaminated surroundings. As controls for this series were taken 10 mice which had been fed 4 months previously on pure-line strains of *B. typhi murium* II without showing any effects or suffering any fatalities, and 40 contact mice in the same series (Q) in which no deaths occurred.[2]

Each mouse received an intraperitoneal injection of 1 cc. of salt solution containing 1:10,000 of an 18 hour agar slant culture of Mouse Typhoid II. This strain had been injected intraperitoneally into mice and recovered twenty-four times, and in doses of 1:30,000 to 1:10,000 regularly caused death of all mice within 200 hours.

All mice in the test except the 10 fed mice were obviously ill within a short time after injection. The results are shown in Table VI.

The results were decisive and indicate that the surviving exposed mice either have not acquired increased resistance to *Bacillus typhi murium* or do not show it by this method of testing. Judging from the manner of spread of mouse typhoid in the replacement experiments from the new to the old mice, of which some had passed through several successive outbreaks, our inference is that

[10] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 71.

no definite protection[11] is accomplished by long survival in epidemics. However, protection was conferred on the 10 mice which had received large numbers of living bacilli with milk.

TABLE VI.

Results of Intraperitoneal Injection of 1:10,000 of an 18 Hour Agar Slant Culture of Mouse Typhoid into Mice Surviving Epidemic Periods R to R 11.

Mice.	No. injected.	No. dead at 18th day.	Per cent dead.
Survivors of epidemic periods R to R 11	128	108	84
Controls.....	40	30	75
Mice previously given large doses of viable single cell strain A by mouth	10	0	0

DISCUSSION.

The results presented in this paper as well as in the first article[2] of this series can be reproduced regularly under the conditions of the experiments, and thus constitute a succession of events to which the term rule may be applied.

It is perhaps desirable to restate in brief just what the events are which have in common the feature of the exposure of successive batches of mice to a disease, mouse typhoid, initiated purposely in a small number of animals through the ingestion of living mouse typhoid bacilli.

In three separate and distinct experimental attempts to induce outbreaks of mouse typhoid resembling the epidemic spreads sometimes witnessed in breeding stocks and thus spoken of as arising spontaneously, the course of the disease produced was of the nature of a sporadic prevalence. That this preliminary outbreak of disease was of this kind is shown not only by the number of mice dying of it but also by the number of cages attacked.

Once, however, this state of sporadic occurrence of mouse typhoid is inaugurated among a mouse population, all that appears necessary in order to convert the occasional deaths with low cage attack rate

[11] Agglutination tests were not made. It is probable that some of the mice possessed blood agglutinins, as it is recalled that in 66 per cent of the tested survivors of the first replacement, the serum agglutinated the epidemic strain.

into frequent deaths with high cage attack rate, is to bring into contiguous relation with the infected population increments of new and healthy mice not previously exposed to the disease.

The particular manner in which the sporadic instances of mouse typhoid arise indicates that very soon a wide distribution of the bacillary incitant takes place in spite of which the fatalities and the cage attack rate remain low.

The sporadic variety of prevalence is soon over. Just what might have taken place subsequently in these lots of mice, had nothing further been attempted, was not determined. No extended observations were made on the survivors, duly segregated, of these preliminary feeding experiments. It can, however, be surmised that under ordinary conditions additional mice would gradually have been added to the population through new births which, in turn, may have become infected, thus reinstating active infection.

But what is definite and significant is the fact that once the sporadic deaths have ceased, the bringing into this population of new lots of mice, which do not mingle directly with the old, suffices to provide the conditions favorable to a wide and even violent outbreak of mouse typhoid, as shown by the fatalities resulting.

The second epidemic spread, or new, in respect to its relative severity and in contrast to the sporadic occurrences, invariably progresses in a certain order. After an interval of about 5 days the new mice begin to succumb, the number of deaths and the proportion of cages attacked rising day by day. During the first period in which the new mice fall victims to the infection, the previously-exposed or old mice do not show an increased death rate. But from the 10th to the 20th day following the addition of the new mice and hence the 5th to the 15th day after the new mice begin to succumb, the old mice are drawn into the wave of fatality with results varying somewhat in the different experiments but in which the old mice finally suffer a mortality to an equal, greater, or less degree.

The epidemic spread of the infection, as indicated by the deaths, tends to subside, and even to disappear entirely, before all, sometimes after only a small part, of the exposed mice have been destroyed. A tendency which is obvious in all the experiments is for the establishment of a state of equilibrium between the surviving mice and

the infecting bacillus. This equilibrium is overcome by the introduction of fresh infectible mouse material, resulting in an outbreak of mouse typhoid. The undulations, or epidemic waves, of the disease thus induced in the different replacements are remarkably uniform. The cessation of the undulation, as indicated by the deaths

TABLE VII.

Number of Mice from Preceding Periods Living at the Time of the Twelfth Replacement Period.

Replacement period.	No. of mice living.	Original No.	Deaths.
			per cent
R 1	23	178	87
R 2	3	228	99
R 3	1	114	99
R 4*	17	41	58
R 5	6	40	85
R 6	6	27	78
R 7	8	30	73
R 8	10	30	67
R 9	17	20	15
R 10	21	30	30
R 11	87	127	31

*Replacements 4 to 10 covered short periods of 6 to 15 days each. The total period covered by these seven replacements was 82 days.

is not abrupt but gradual, although the late fatalities may not be due to delayed infection so much as to protracted illness and long survival.

The experiments show definitely that mice which have weathered the storm of the epidemic in the sense of having passed, without succumbing, through one or more violent outbreaks of the disease, are not insured against eventual fatal attack. This fact is brought out in Table VII, which shows that as successive epidemic waves pass over the always changing population, the old or previously exposed mice tend ultimately to be wholly wiped out.

In due time the precise conditions concerned in the conversion of the incipient and sporadic into epidemic spreads of disease will need to be considered narrowly. We have learned in respect to the enteric infection mouse typhoid how the one may be turned into the other

and that the epidemic outbreaks are always, as it were, frustrated before all the available infectible material is consumed.

Obviously there are two outstanding factors which affect and determine the state of the microbic incitant in its relation to the host: the one relates to the quality or virulence, so called, the other to the quantity or number. Hitherto it has been the virulence factor that has been most considered and invoked to account for the wave-like movement of epidemic disease. This fact will appear prominently in the brief review of the literature which is to follow. The second or quantity factor has been much less discussed. As pointed out in the body of this paper, we believe that it cannot well be disregarded. Perhaps the disease mouse typhoid may provide favorable experimental materials for just this study. Our experiments show decisively that immediacy of contact is by no means a necessary condition for the wide diffusion of the microbic incitant of mouse typhoid among a mouse population maintained in small segregated groups; and this fact is emphasized also by the occurrences of the spontaneous epidemic described by Lynch.[1] The mere rapidity of the fluctuation of the curve of the death rate in the replacement experiments either indicates that the virulence factor is highly unstable, or that some other attending condition acts to modify or control the results as measured by infection and death.

Thus far attention has centered on the factors affecting the microbic incitant, while those which may be presented by variation in the host have yet to be considered. Difficult as may be the unravelling of the former, for which certain methods possibly are at hand, the other has been regarded next to insuperable. Aside from choosing a stock roughly homogeneous and covering the matter of size and age, nothing has been attempted as regards the host. In a recent study[12] of tuberculous infection in guinea pigs an effort has been made to obtain values for certain hereditary qualities, an aspect of the problem of infection in mouse typhoid capable of treatment.

The relation of the carrier state to the host awaits elucidation. That carriers arise among the exposed mice which do not succumb early is demonstrated. Experience indicates that the detection

[12] Wright, S., and Lewis P. A., *Am. Naturalist*, 1921, lv, 20.

of carriers is determinable not by cultivation tests alone but chiefly through the reactions of immunity. Without the latter, error readily creeps into the results because of the presence in the intestine of apparently normal mice, of members of the broad group of organisms embraced under the loose term of mouse typhoid bacilli.[10] Webster's experiments show that the purposive introduction of living or even killed mouse typhoid bacilli into the intestines of normal mice, under proper conditions, leads to an immunization sufficing to prevent infection from otherwise fatal infecting doses of cultures for such mice. This state of immunity is general in the sense that specific agglutinating bodies are demonstrable in the blood, and the immunized mice resist not only intrastomachic but also intraperitoneal injections of the living bacilli.

Review of the Literature.

The possibility of employing artificial epidemics among small rodents for the study of the spread of infection should date from the experiments of Danysz,[13] published in 1900. As will be recalled, he endeavored to exterminate rodents, rats especially, on a large scale through the employment of living cultures of a bacillus belonging to the so called mouse typhoid group. The method did not really succeed. The reasons for the failure are now apparent and, in the main, arise first from the rapid loss of infecting power or virulence of the cultures, and second from the circumstance that the bacillus as it occurs in the rat cadaver is less infective than at the moment infection takes place. Hence the devouring of the cadavers by their living companions arrests rather than propagates the disease. These unsuccessful results were foreshadowed by laboratory studies made by Danysz, who found that no matter how highly active the cultures were at the outset, their power of infection by feeding did not extend beyond a very few passages. Danysz' conclusions may be stated as follows:

Cultures may be enhanced so that they will induce fatal infection on ingestion, but the increased activity will not endure beyond the third or fourth passage; exposure of a group of non-infected mice to a small number of mice purposely fed on a virulent culture will induce fatal infection in most but not in all of the former. The few survivors succumb to a later direct feeding of culture of average virulence. Cultures enhanced by organ and blood passages suffer concomitant diminished activity when administered *per os*. Cultures obtained from the blood and organs in the incubation stage of infection are more virulent than when derived

[13] Danysz, J., *Ann. Inst. Pasteur*, 1900, xiv, 193.

from cadavers. Resistance or susceptibility to infection among small rodents is affected by race and age, and even by individual peculiarities.

Later, Bainbridge[14] noted that a spontaneous epidemic among wild rats kept under experimental conditions may lead to the death of all, and as the conditions approach those of nature, the death rate becomes less. The percentage of deaths noted by Bainbridge was not affected by the number of rats devoured, and he considered the rat to possess a preexisting immunity which arises through the occurrence of spontaneous epidemics among those animals. In support of this view, rats surviving the feeding of cultures were found to carry agglutinating properties in their blood for the bacilli used.

The conditions favoring the perpetuation of the pathogenic proclivities of the mouse typhoid bacilli have been investigated recently by Topley. He has utilized his material, as we had planned to use ours, in an endeavor to penetrate into the hidden recesses of epidemiology. He repeated Danysz' experiments, which he confirmed, but he went beyond the latter in acting on the supposition that the consumption of food soiled by excreta may be a more common and effective mode of infection than the devouring of cadavers carrying already depressed bacilli.

From the first, Topley[15] looked upon the spread of epidemics and their rise and fall as having to do primarily and essentially with the infecting power of the inciting microbe. The rapid fluctuations of the mouse typhoid bacillus in this regard seemed to provide the needed explanation of the failure to presage epidemics among mice. He sought to supply the needed conditions by starting an epidemic through feeding and then adding, in a large cage, daily small complements of normal mice, in order, through passage, to keep the bacilli alive and actively infective. In this way the tendency for the disease, once started, to cease abruptly was overcome, and the infection would proceed in regularly recurring waves, apparently without time limit. He further observed that the deaths occur in large groups with intervals between, during which the deaths are few or far between. He distinguished two phases according to which the new mice added tended to succumb or survive, and these phases are the rise of a wave when infection is likely to occur and its fall when it is likely to fail. Finally, he noted that mice which have passed successfully through one epidemic wave may succumb to a later one, and an infected population to which no additions are made will outlive one into which fresh mice are brought, while the ultimate survivors have not escaped infection and a considerable part has become carriers. A substitution of strains took place during Topley's experiments as in ours. He began with a strain of Gärtner's bacillus, but as his experiments progressed a second organism, identified as belonging to the suipestifer group, intervened. Subsequently strains

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[14] Bainbridge, F. A., *J. Path. and Bact.*, 1909, xiii, 443.

[15] Topley, W. W. C., *J. Hyg.*, 1920-21, xix, 350.

of Gärtner's bacillus, or of this second organism, or both together, were obtained from the great majority of the dead animals examined.[16]

In a second communication[17] the outbreak of an epidemic among a normal stock, probably through accidental spread from cages with infected mice, is described. Not all the exposed mice succumbed and the deduction made was that the infection was not of "uniform intensity, passed from a healthy carrier to one or more susceptible individuals, but that some process is set in motion, which results in an increase in the infectivity of the parasite, this in its turn giving rise to a fresh wave of mortality among the cage population."

Topley's experiments were arranged to achieve the greatest likelihood of infection occurring by providing the greatest possible concentration of contaminated materials; namely, dejecta carrying the bacilli or cadavers devoured by their companions. Our experiments were long under way before Topley's main publications appeared in 1921. We had in mind not the providing of optimal conditions for infection to take place in mice, but the imitation, if only roughly, of those occurring naturally in man and in laboratory animals, in connection with which epidemics of disease occur.

SUMMARY.

1. A kind of mouse village was set up into which was introduced a small number of mice fed on a culture of so called mouse typhoid (*Bacillus pestis caviæ* of the *Bacillus paratyphosus* B group) bacillus. The spread of the infection so induced to the cages, or "homes," of the other mice was left to accident through the attendant who fed the animals and cleaned the cages. That this means was likely to be sufficient was deduced from the epidemic reported by Lynch.[1] A spot map was kept throughout the experiments which extended from 1919 into 1922.

2. The first effect of the exposure of normal mice to a much smaller number of mice fed on the culture is to set up a *sporadic*,

[16] Topley and his coworkers (Topley, W. W. C., Weir, H. B., and Wilson, G. S., *J. Hyg.*, 1921, xx, 227) have studied these two organisms serologically, along with other related bacilli, and suggest that the relations between the Gärtner's bacillus and *B. suipestifer* group of organisms is so close that for purposes of classification and nomenclature the name of *B. enteritidis* may, for convenience, be applied to the whole group.

[17] Topley, W. W. C., *J. Hyg.*, 1921, xx, 103.

not an *epidemic* outbreak of mouse typhoid. This is the regularly recurring incident of the experiment as shown by low mortality and low cage attack rate. Such a sporadic prevalence is self-limited in time.

3. The introduction of fresh, normal mice into a community in which sporadic deaths are occurring leads regularly, not to the further extension of the sporadic deaths, but to an epidemic spread, as shown by high mortality and high cage attack rate. The epidemic begins with deaths among the new mice, but extends to the old mice which succumb later. The spread ceases and the wave subsides before all the mice have succumbed. A state of equilibrium between the infecting bacillus and the surviving mice is reached; no more deaths occur. The epidemic outbreak, therefore, is also self-limited in time.

4. If, now, another new addition of normal mice is brought into the potentially infected community, the events are reenacted; deaths occur among the new, another epidemic wave sweeps through the population, again claiming victims among the previous survivors.[18] Through the replacement of the destroyed mice with fresh, normal mice, epidemic wave after wave is produced, until certain groups of old survivors are entirely wiped out. There seems to be no limit to this process, as there will always be survivors at least of the later groups added.

5. The dying down of the epidemics and the attaining of the equilibrium do not mean the elimination of all the bacilli. Potential infection still lurks in mouse "carriers" and on the hands of the attendant. No successful method of completely removing the bacilli from the hands of the attendant was found.

6. The maintaining of the epidemic waves is dependent on the presence of new lots of mice, whether supplied from without or produced within through new births. It is the latter sources which provide the consumable material in such natural epidemics as that described by Lynch.[1] Hence their slower movements as compared with the epidemic spreads and rise and fall of the epidemic waves in the artificially propagated instances in which new batches of mice are brought into the village in bimonthly intervals.

[18] In the third replacement period a rise in death rate constituting a small epidemic occurred after the 60th day and independent of the addition of new mice. No explanation has been found for this occurrence.

7. The evidence at hand is to the effect that the degree of infectivity of "mouse typhoid" bacilli is highly fluctuating, and it appears that all the bacilli which are included under that name, classed variously as *Bacillus enteritidis*, Gärtner's bacillus, *Bacillus paratyphosus* B, *Bacillus suipestifer*, and *Bacillus pestis caviæ*, infect mice in a similar, possibly indistinguishable manner, inducing self-limited outbreaks of disease reaching at times epidemic proportions. This quality of infectivity, or virulence, is one factor in the process but alone does not suffice to account for the observed facts. A second influence is not improbably quantity, or dosage, of the inciting microorganism. Attention to the manner of spread of an epidemc, as revealed graphically by the spot map, shows that it is never uniform but patchy. The supposition is, therefore, that among the new mice are certain individuals so highly susceptible as to react to small numbers of bacilli of average infectivity. Within these animals the multiplication is rapid, so that a wider spread of much larger amounts of these average, or even temporarily enhanced bacilli takes place with the inevitable effect of communicating, through greater dosage, etc., the infection to other and less susceptible individuals among the new and also the older lots. This process continues so long as rapid multiplication can occur. This latter is, in turn, determined by the innate tendency of the bacilli to return to an average of infectivity and by mouse individuals of greater resistance to restrict free growth and multiplication. In this manner, possibly, the epidemic spread is checked and the curve representing it made to fall more or less quickly to a base-line.

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EXPERIMENTS ON NORMAL AND IMMUNE MICE WITH A BACILLUS OF MOUSE TYPHOID.

BY LESLIE T. WEBSTER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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As part of a wider study of the phenomena of epidemics among animals described by Flexner[1] and Amoss,[2] the following series of experiments was undertaken to ascertain the varieties and degrees of resistance in normal and immunized mice to mouse typhoid bacilli of the paratyphoid-enteritidis group. The experiments were so designed as to bear directly on such questions as the relation of infectivity on the part of the microorganisms to the portal of entry into the body of the host and also on the influence of a possible local as opposed to a general immune state, the effect of which would be to alter or even to abate the danger of infection by the ordinary route traversed in nature.

Since the problem of infection, as manifested in the higher animal species, is a complex made up of potentialities in the host and in the microbe, it becomes necessary to control and keep constant as many of the factors as is possible. Hence the mice, chosen from healthy stock with practically no death rate, were of one age, often from the same litter, of practically the same weight, and were kept under close observation before and during experiments. The stock room diet of bread and milk was continued throughout the experiments. The mouse typhoid strain, M. T. II, obtained from an experimental epizootic studied by Amoss,[2] was identified with *Bacillus pestis caviæ* Smith and closely related to *Bacillus aertrycke* (mutton) of Schütze.[3] This organism was carried in stock and maintained by monthly stab cultures in tube agar of pH 7.4. In order to secure a culture of

[1] Flexner, S., *J. Exp. Med.*, 1922, xxxvi, 9.

[2] Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25, 45.

[3] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 97.

Strain M. T. II of a degree of virulence adequate to infect regularly *per os*, it was necessary to pass it through a series of mice by intrastomachal inoculation. At autopsy, to guard against substitution, the identity of the recovered bacilli was always established by means of fermentation and agglutination tests. Dosage was determined by making use of the fact that cultures of Strain M. T. II in plain broth of pH 7.4, when inoculated in small amounts, reached, after 14 to 24 hours incubation at 37°C., a relatively constant number; *e.g.*, 1 billion per cc. With a 14 to 24 hour broth culture the approximate number of organisms contained in any dilution could thus be calculated.

Effects on Normal Mice of Intrapleural, Intraperitoneal, and Intrastomachal Inoculations.

Intrapleural Inoculation with Culture M. T. II.—These experiments and others to follow were planned in order to ascertain the manner of resistance of mice to intrapleural injections of the cultures. The tests were made as follows:

9 mice with controls were given intrapleurally 0.2 cc. of a 16 hour broth culture of Strain M. T. II obtained from the stock agar tube. Interpreting these and all subsequent dilutions on the 1 cc. basis, 3 mice received 1:5,000 dilution, 3 received 1:50,000 dilution, and 3 received 1:500,000 dilution. At 2 hour intervals small amounts of fluid were aspirated from the infected pleural cavity. Films were made and 1 drop was transferred into fluid agar (48°C.), shaken, and poured. Colonies were counted after 24 hours incubation (37°C.). Table I summarizes the duration of life of experimental animals; the controls ran a similar course.

TABLE I.

Duration of Life of Mice Injected Intrapleurally with Strain M. T. II.

Dilution.	Duration of life in Experiment 2.	Duration of life in Experiment 3.	Duration of life in Experiment 4.	Average duration of life.
	days	*days*	*days*	*days*
1:5,000	5–1–3	1–6–3	1–1–3	2+
1:50,000	5–5–6	Survived*–3–5	4–10–7	5+
1:500,000	6–7–5	15–9–14	10–6–6	8+

* Survivors were discarded after 30 days.

The counts of the colonies on the plates, although not quantitatively accurate, were, nevertheless, of value in showing the relative changes in numbers. Such sources of error as dry puncture, irregularity of distribution of bacilli in the pleural exudate, and variation in size of drop, did not obscure the tendency of the bacteria after an initial lag of 4 to 6 hours to increase rapidly in number until the mice were overwhelmed (Table II and Text-fig. 1).

TABLE II.

Bacterial Growth in Vivo (Intrapleural).

Dilution 1:50,000; number of colonies in plate 150 to 200.

Length of time after injection.	Mouse 1.				Mouse 2.				Mouse 3.			
	Condition.	Smear.		No. of colonies in plates.	Condition.	Smear.		No. of colonies in plates.	Condition.	Smear.		No. of colonies in plates.
		Cells.	Phagocytosis.			Cells.	Phagocytosis.			Cells.	Phagocytosis.	
hrs.												
1	Well.	±	0	100	Well.	±	0	30	Well.	±	0	60
2	"	±	0	100	"	±	0	50	"	±	0	100
3	"	±	±	50	"	0	0	50	"	0	0	40
4	"	±	+	100	"	0	0	100	"	0	0	100
5	Sick.	+	+	150	Sick.	+	+	250	Sick.	0	0	15*
6	"	+	?	250	"	+	+	250	"	?	?	250
24	"	++	+	750	"	+	+	250	"	+	?	150
48	"	++	+	750	"	+	?	250	"	?	?	250
72	"	++	+	1,200	"	++	+	720	"	+	+	450
98	"	?	?	1,620	"	+	?	690	"	+	+	2,000
119	Dead.				Dead.							
143									Dead.			

* Probably an error.

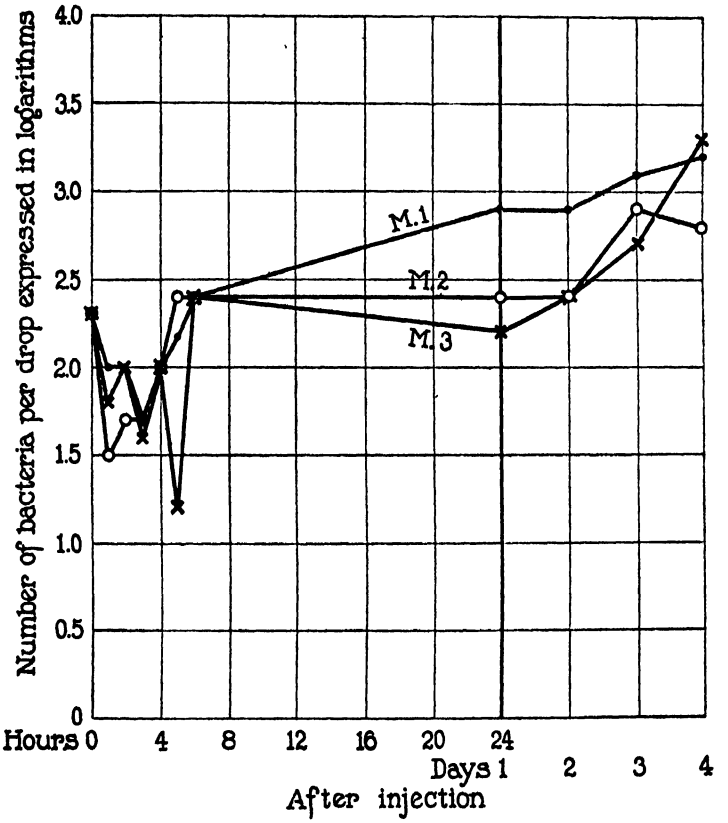
The series of events following the intrapleural injection of fixed doses of a constant culture was quite regular, as was shown by repetition of the experiment just described.

Intraperitoneal Inoculation with Culture M. T. II.—This experiment was the counterpart of the pleural one immediately preceding. It was carried out as follows:

6 mice with controls were injected intraperitoneally with 0.2 cc. of a 16 hour broth culture of Strain M. T. II obtained from the stock agar tube, 3 receiving 1: 5,000 and 3, 1:50,000 dilution. At short intervals small amounts of fluid were

aspirated from the peritoneal cavity. Films were made and 1 drop of the exudate was plated in the fluid agar (48°C.) medium. The colonies were counted after 24 hours incubation (37°C.).

The result is similar (Table III); after an initial lag, the bacilli multiply rapidly until they reach a high number at about the time death occurs, as is shown in Text-fig. 2.



TEXT-FIG. 1. Bacterial growth *in vivo* (intrapleural). The figures are taken from Table II.

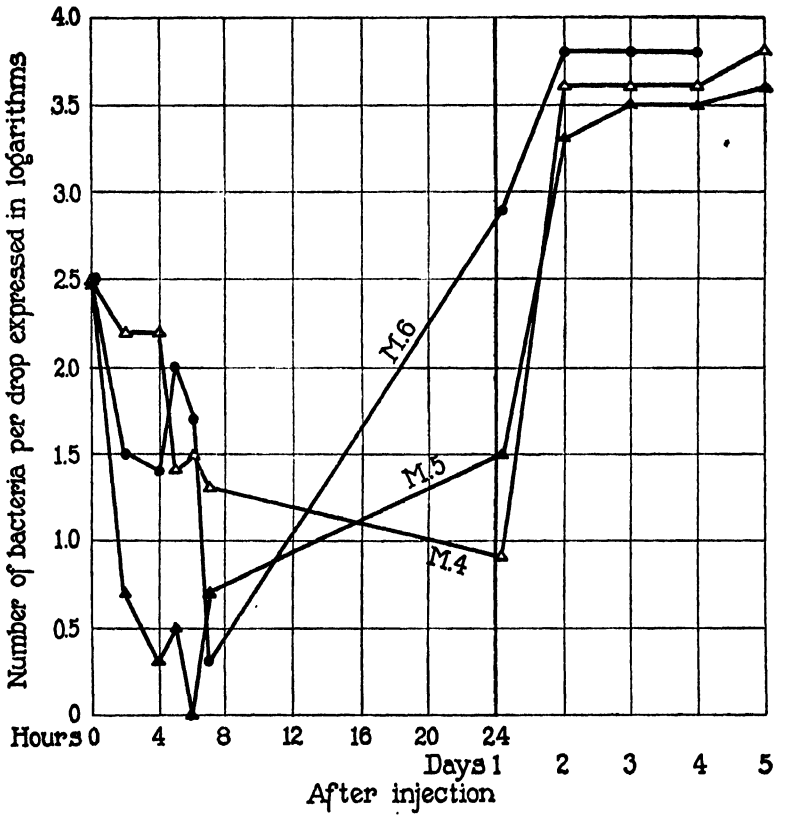
The events in the two experiments with pleural and peritoneal inoculation can be summarized as follows: if live cultures of this organism are injected intrapleurally or intraperitoneally into normal mice, there occurs an initial lag in the rate of bacterial multiplication lasting 4 to 6 hours, followed by a rapid and continual acceleration of

TABLE III.

Duration of Life of Mice Injected Intraperitoneally with Strain M. T. II.

Dilution.	Duration of life in Experiment 9.	Duration of life in Experiment 10.	Average duration of life.
	days	*days*	*days*
1:5,000	6–3–5	10–10–Survived.*	7
1:50,000	7–6–5	13–5–13	8+

* Survivors were discarded after 20 days.



TEXT-FIG. 2. Bacterial growth *in vivo* (intraperitoneal).

growth until the death of the animal. To this rule there are exceptions in an occasional recovery or an undue prolongation of the survival period. The mice dying acutely exhibit few pathological changes,

while those in which the infection pursues a more chronic course show macroscopically thick peritoneal or pleural exudation, and lesions of the lungs, liver, and spleen consisting of hepatization, nodule formation, swelling, etc. The injected bacillus was always recovered from the heart's blood and organs.

Intrastomachal Inoculation with Culture M. T. II.—A fixed dose which ranged from 0.5 to 1 cc. of the broth culture diluted 1:100 was injected into the stomach through a stiff silver catheter attached to a suitable measuring syringe with rubber tubing. This slight procedure which assures ingestion of the culture can be carried through expeditiously and securely. Culture M. T. II could be kept at an approximately constant infecting level if passed through mice *per os* at frequent intervals. The injections *per os* were made about 3 hours after feeding.

Thus far, in spite of the control exercised over the origin and selection of the mice and the uniform treatment and dosage of the culture, some degree of fluctuation has attended the experiments in infection of normal mice *per os*. That irregularities would enter into this part of our studies was of course foreseen. It is highly probable that under ordinary conditions of propagation of an epizootic among mice by a representative of the paratyphoid-enteritidis group, infection takes place always *per os*, and yet we know that survivors invariably occur. Our experiments show that the survivors have no necessary relation to dosage of a given constant culture, although on the whole the animals receiving the larger doses are the ones tending to succumb. Table IV is intended to bring out the variations met with in a number of carefully planned tests.

In addition to the irregularities shown in these experiments, it happens that still greater ones are met with when instead of introducing the culture directly into the stomach of mice, the organisms are added to the food by soaking up the broth culture with bread which is then fed to the animals.

For this test the mice were assembled 6 per cage, and it was noted that they devoured the soaked bread voraciously on successive days without necessarily succumbing to infection. Table V summarizes the results of two such feeding experiments.

TABLE IV.

Variation in Susceptibility Following Injection per Os (Stomach Tube).

No. of mice.	Broth dilution.	Duration of life in fatal cases.	No. of survivors.	Result.
		days		
1	1:100	9	0	
2	1:200	12–16	0	Regular.
1	1:500		1 (60 days).	
3	1:100	8–14–20	0	
1	1:1,000		1 (60 days).	Regular.
1	1:10,000		1 (60 ").	
3	1:100	19–21	1 (60 ").	
1	1:1,000		1 (60 ").	Regular.
1	1:10,000		1 (60 ").	
3	1:50	12–43	1 (60 ").	
3	1:500	11	2 (60 ").	Irregular.
3	1:5,000	17–18–23	0	
3	1:50,000		3 (60 days).	
2	1:1	18	1 (30 ").	
3	1:10	8–11–21	0	Irregular.
3	1:100	7–8	1 (30 days).	
2	1:1	4–10	0	
2	1:10		2 (18 days).	Irregular.
2	1:100	17–18	0	

TABLE V.

Variation in Susceptibility Following Injection per Os of Strain M. T. II (with Feedings).

No. of mice.	Duration of feeding.	Broth dilution.	Duration of life in fatal cases.	No. of survivors.	Result.
	days		*days*		
3	3	1:10,000	12	2 (30 days).	
3	5	1:10,000		3 (30 ").	Irregular.
3	7	1:10,000	17	2 (30 ").	
5	5	1:10,000		5 (40 ").	
5	7	1:10,000	11–11–17	2 (40 ").	Irregular.
5	9	1:10,000	15	4 (40 ").	
5	13	1:10,000	22–38	3 (40 ").	

Although the stomachal injections and the feedings of broth cultures of Strain M. T. II did not consistently induce infection but rather brought out factors of variation, themselves illuminating when considered in relation to observed epizootics arising accidentally among mice, yet certain general features may be gleaned from the tests performed. An incubation period of about 5 days preceding the indications of infection or death was noted. There was no invariable relation between dosage and fatal infection; without reference to dosage or culture certain mice proved refractory. The type of infection was predominatingly chronic with regular involvement of the intestine, spleen, and liver, and occasional involvement of the lungs and heart. In all instances Strain M. T. II was recovered from the heart's blood and organs.

Effects on Immune Mice of Intrapleural, Intraperitoneal, Subcutaneous, and Intrastomachal Inoculations.

The next tests related to the influence exerted by immunizing injections of dead or living bacilli on the subsequent inoculation of given doses of active cultures of Strain M. T. II. The experiments were arranged to bring out the effect not only of the immunizing action in general immunity but also of a possibly greater state of local immunity at the site of the protective inoculations. The live organisms were introduced into the pleural and peritoneal cavities and also into the stomach.

The killed cultures employed for the protective injections consisted of a 24 hour broth culture which had been heated to 55°C. for 2 hours and afterwards standardized and preserved with 0.3 per cent tricresol.

Intrapleural and Intraperitoneal Inoculation.—The killed bacilli, in doses of 250,000, were injected either into the right pleural or the peritoneal cavity, after which, at the expiration of a given period of time, living bacilli were injected into one or the other of these cavities, as shown in the protocols which follow.

12 mice were inoculated with 250,000 killed Strain M. T. II bacilli, 6 intrapleurally and 6 intraperitoneally, and 7 days later the inoculations were repeated. 9 days after the second inoculation the mice were divided into batches of 3 and injected with 0.2 cc. of a 1:1,000, 14 hour broth culture of Strain M. T. II either intrapleurally or intraperitoneally. 3 pleural "vaccinated" mice, along with 3

normal controls, were injected intrapleurally, another 3 of the pleural "vaccinated" mice, together with 3 controls, received the material intraperitoneally. At intervals, fluid was aspirated from the cavity into which the living bacilli were injected, films were made, and 1 drop was plated in agar. The colonies developing in the latter were counted after 24 hours growth. Tables VI to VIII inclusive and Text-fig. 3 give in brief the results of the experiment.

TABLE VI.

Duration of Life of Normal and "Vaccinated" Mice Injected Intrapleurally and Intraperitoneally.

Mice.	Mice "vaccinated" intrapleurally; injected intrapleurally.		Mice "vaccinated" intrapleurally; injected intraperitoneally.	
	Duration of life.	Average duration of life.	Duration of life.	Average duration of life.
	days	*days*	*days*	*days*
"Vaccinated".....	13–14–28	18+	14–17–25	18+
Controls.....	2–4–4	3+	1–1–5	2+

TABLE VII.

Bacterial Growth in the Pleural Cavities of Normal and "Vaccinated" Mice. Mice "Vaccinated" Intrapleurally; Injected Intrapleurally.

Length of time after injection.	14 hr. broth culture of Strain M. T. II; dilution 1:5,000; 5,000 organisms per drop.					
	Mouse 1, "vaccinated." No. of colonies in plates.	Mouse 2, "vaccinated." No. of colonies in plates.	Mouse 3, "vaccinated." No. of colonies in plates.	Mouse 4, control. No. of colonies in plates.	Mouse 5, control. No. of colonies in plates.	Mouse 6, control. No. of colonies in plates.
hrs.						
2	2	0	1	100	420	120
3	0	0	0	300	150	300
5	0	0	5	450	600	60
6	3	0	3	450	180	800
8	0	0	1	450	3,600	720
9	0	0	0	4,000	5,400	3,000
30	120	0	30	2,000	900	800
days						
2	0	0	10	5,000 Dead.	100	1,800
3	0	3	2		360	Dry tap.
4	180	40	10		Dead.	Dead.
5	240	25	150			
11	1,620	1,080	1,350			
13	Dead.					
14		Dead.				
23			1,440			
28			Dead.			

TABLE VIII.

Bacterial Growth in the Peritoneal Cavities of Normal and "Vaccinated" Mice. Mice "Vaccinated" Intrapleurally; Injected Intraperitoneally.

Length of time after injection.	14 hr. broth culture of Strain M. T. II; dilution 1:5,000; 5,000 organisms per drop.					
	Mouse 7, "vaccinated." No. of colonies in plates.	Mouse 8, "vaccinated." No. of colonies in plates.	Mouse 9, "vaccinated." No. of colonies in plates.	Mouse 10, control. No. of colonies in plates.	Mouse 11, control. No. of colonies in plates.	Mouse 12, control. No. of colonies in plates.
hrs.						
2	90	1	10	360	3,600	80
3	0	0	15	3	1,350	550
5	4	0	10	40	720	15
6	1	0	1	120	1,080	1,350
8	0	0	0	3,150	4,000	6,000
9	0	2	0	4,000	6,000	6,000
30	15	0	30	4,000	Dead.	Dead.
days						
2	20	0	Dry tap.	4,000		
3	0	15	" "	6,000		
4	150	360	" "	6,000		
5	180	90	" "	Dead.		
11	6	3,000	" "			
14		Dead.				
17	Dead.					
23			15			
25			Dead.			

TABLE IX.

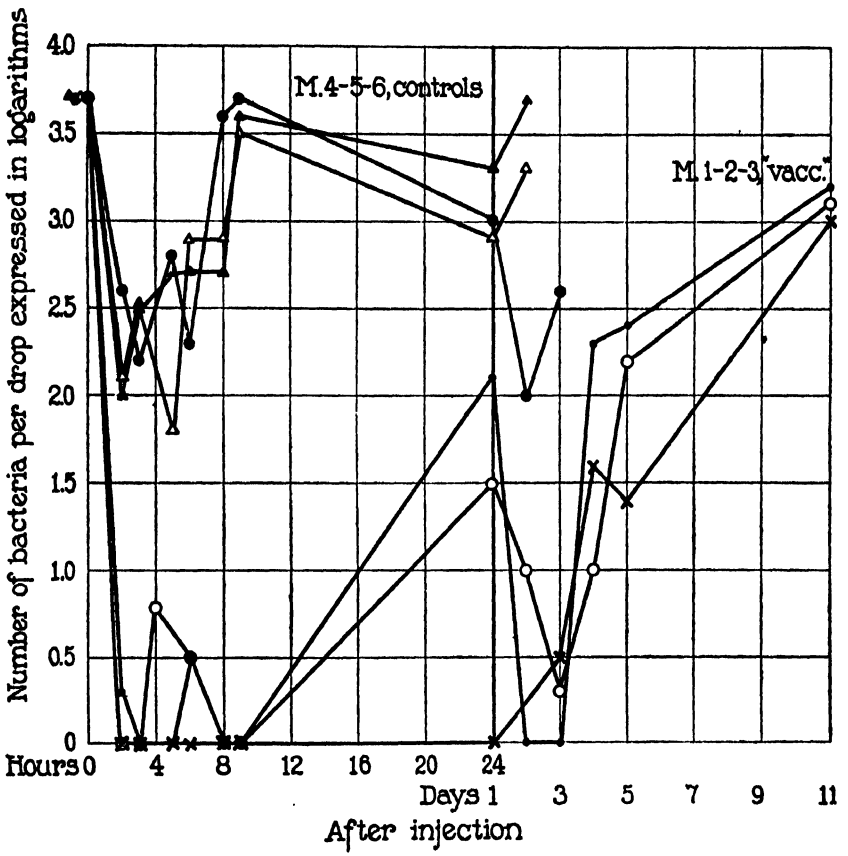
Duration of Life of Normal and "Vaccinated" Mice Injected Intrapleurally and Intraperitoneally.

Mice.	Mice "vaccinated" intraperitoneally; injected intrapleurally.		Mice "vaccinated" intraperitoneally; injected intraperitoneally.	
	Duration of life.	Average duration of life.	Duration of life.	Average duration of life.
	days	*days*	*days*	*days*
"Vaccinated."	15–17–19	17	21–36–Survived.*	32+
Control.	4–4–1	3	3–4–4	3+

* Survivors were discarded after 40 days.

This experiment was paralleled by a similar one in which the "vaccination" had been made intraperitoneally, followed as before by the injection of living bacilli into the pleural or peritoneal cavity. The results in general were identical

with the preceding: Table IX shows the duration of life in the series and the curves illustrating bacterial growth in "vaccinated" mice and controls are similar to those of Text-fig. 3.



TEXT-FIG. 3. Bacterial growth in the pleural cavities of normal and "vaccinated" mice.

The experiments may be summarized as follows: Living bacilli injected into the control mice showed the usual lag of 4 to 6 hours, followed by a rapid increase in number until the death of the animal. These mice succumbed within 3 days after injection, showed few lesions at autopsy, and positive cultures from the heart's blood and organs. Living bacilli injected into the "vaccinated" mice were partially destroyed and held in check by the protective mechanism of

the animal body for 2 or 3 days. Subsequently the rate of bacterial multiplication increased gradually until the death of the animal. These mice succumbed within 18 days after injection, and showed at autopsy extensive local lesions as well as marked involvement of the spleen and liver. Cultures from the heart's blood, exudates, and organs were positive. The partial immunity following this type of treatment is entirely of a general nature; no essential difference in the progress of infection or ultimate protection is to be noted whether the mice are given the injection into the cavity which previously received the "vaccine" or into the cavity previously "unvaccinated."

Subcutaneous Inoculation.—

As a corollary to the above, 6 mice were "vaccinated" subcutaneously with a total of 150,000,000 bacilli given in two doses. 5 days after the second inoculation, twice the usual dose of living bacilli was given to 3 of the mice intrapleurally, 3 intraperitoneally, and to normal controls. Because of the larger dose of living bacilli, the controls died within 24 hours, while the average survival period of the "vaccinated" mice was 17 days. The progress of the infection, however, was to all intents and purposes identical with that in the mice "vaccinated" by way of the pleura and peritoneum and their controls.

Inoculation per Os.—The ordinary, or as we say, natural mode of infection with mouse typhoid bacilli is by way of the gastrointestinal tract and hence the influence of the "vaccination" on infection *per os* was next studied. Since the preceding tests all indicate that the effects of protective inoculation with killed cultures on the progress of a subsequent injection of living bacilli are practically identical, however the "vaccination" is carried out, namely by the pleural, peritoneal, or subcutaneous route, the inoculation of dead bacilli in this experiment was made subcutaneously.

150 normal mice of approximately the same weight (16 to 18 gm.) and age (2 months) were segregated. To 120 of them 0.5 cc. of a 24 hour broth suspension of Bacillus M. T. II, previously heated to 55°C. for 2 hours, was given under the skin. The "vaccine," preserved with 0.3 per cent tricresol, was standardized to contain 500,000,000 bacilli per cc., so that each mouse received an initial dose of approximately 250,000,000 dead bacilli. 30 mice were set aside to be used as controls.

After an interval of 13 days, 10 of the mice having received one injection of the "vaccine" were given an intraperitoneal injection of 0.5 cc. of a living 14 hour broth culture of Mouse Typhoid II, diluted 1:100, and a second 10 of the "vaccin-

ated" mice received the same dose into the stomach by silver tube. Controls were injected intraperitoneally and *per os* with doses of 0.5 cc. of the same broth culture diluted 1:50 to 1:250.

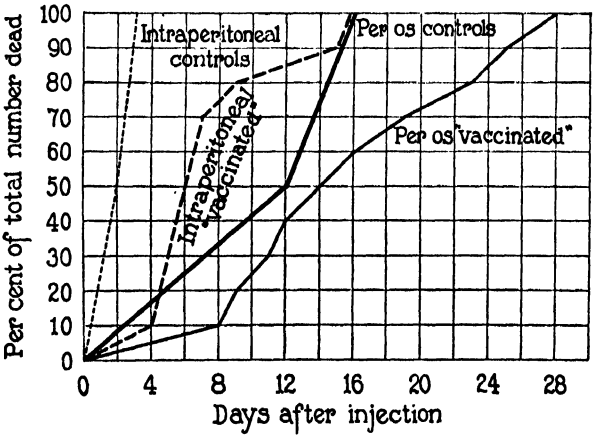
TABLE X.

Duration of Life of Normal and "Vaccinated" Mice Injected Intraperitoneally and Intrastomachally.

Mice.	No. of mice.	Dosage.	Duration of life.	Average duration of life.
			days	*days*
Intraperitoneal control.	1	1:100	1	1
" "	2	1:200	2–3	2.5
" "	1	1:500	4	4
" "vaccinated."*	10	1:200	4–5–5–6–6–7–7–9–15–16	8
Per os control.	1	1:100	9	9
" " "	2	1:200	12–16	14
" " "	1	1:500	Survived.†	
" " "vaccinated."*	10	1:200	8–9–11–12–14–16–19–23–25–28	16.5

* The "vaccinated" mice had received one subcutaneous injection previous to the intraperitoneal or intrastomachal injection.

† Survivors were discarded after 62 days.



TEXT-FIG. 4. Duration of life of "vaccinated" mice injected intraperitoneally and intrastomachally. The figures are taken from Table X.

Table X and Text-fig. 4 summarize the results of this experiment, which can be expressed as follows: A single subcutaneous inoculation

of killed bacilli into mice confers a partial protection against subsequent introduction of live organisms *per os* as well as by way of the peritoneal cavity.

The remaining 100 "vaccinated" mice received a second subcutaneous injection of the killed cultures 14 days after the first. After the expiration of another 18 days, 20 of these mice were given 1 cc. of a 14 hour living broth culture of Strain M. T. II diluted 1:100 into the peritoneum, and 20 the same dose by stomach tube. Table XI and Text-fig. 5 summarize the results of the injections in these animals and in the controls.

TABLE XI.

Duration of Life of Normal and "Vaccinated" Mice Injected Intraperitoneally and Intrastomachally.

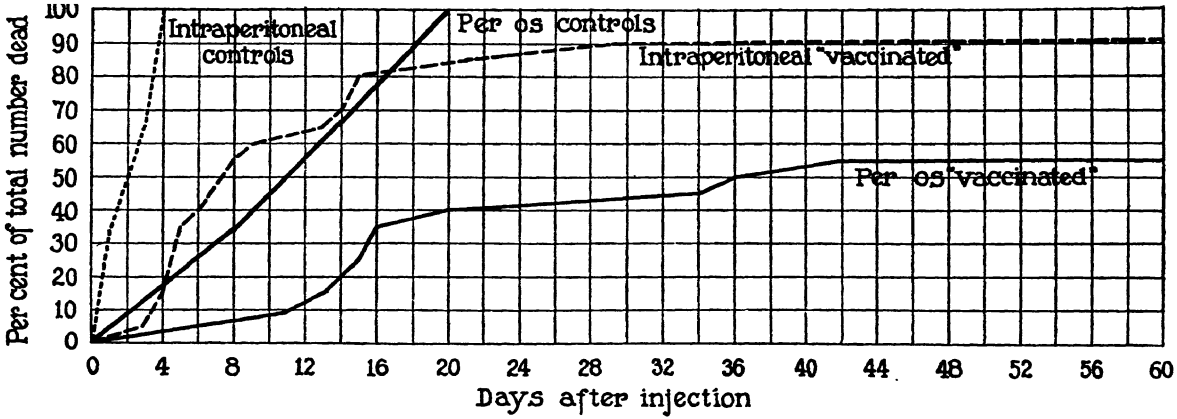
Mice.	No. of mice.	Dosage.	Duration of life.	Average duration of life.
			days	*days*
Intraperitoneal control.	3	1:100	1–3–4	2.6
" "	1	1:1,000	5	5
" "	1	1:10,000	5	5
" "vaccinated."*	20	1:100	3–4–4–5–5–5–5–6–8–8–8–9–13–14–15–15–21–29. Two survivors.†	10
Per os control.	3	1:100	8–14–20	14
" " "	1	1:1,000	Survived.†	
" " "	1	1:10,000	" †	
" " "vaccinated."*	20	1:100	11–11–13–15–15–16–16–20–34–36–42. Nine survivors.†	20

* The "vaccinated" mice had received two subcutaneous injections previous to the intraperitoneal or intrastomachal injection.

† All survivors were discarded after 62 days.

The increase in partial protection following a second injection of "vaccine" is effective against the *per os* as well as the peritoneal portal of entry.

40 of the remaining "vaccinated" mice were given 25 days later a third subcutaneous injection of 250,000,000 killed bacilli, of which, after the lapse of another 10 days, 20 were injected into the peritoneum and 20 by stomach tube with the regular dose of 1 cc. of a 14 hour living broth culture diluted 1:100.



TEXT-FIG. 5. Duration of life of "vaccinated" mice injected intraperitoneally and intrastomachally. The figures are taken from Table XI.

The results are given in Table XII and differ from those in Table XI only in the number of survivors among the "vaccinated" animals, which is much larger. It must be noted, though, that the increased protection from the third "vaccination" is somewhat more effective against the introduction of live organisms intraperitoneally than by way of the mouth.

The results of these experiments show that mice "vaccinated" subcutaneously with a certain mouse typhoid strain are partially

TABLE XII.

Duration of Life of Normal and "Vaccinated" Mice Injected Intraperitoneally and Intrastomachally.

Mice.	No. of mice.	Dosage.	Duration of life.	Average duration of life.
			days	*days*
Intraperitoneal control.	3	1:100	1–1–1	1
" "	1	1:1,000	1	1
" "	1	1:10,000	5	5
" "vaccinated."*	20	1:100	7–8–8–10–10–12–12–13–15–16–17–18–32–33. Six survivors.†	15+
Per os control.	3	1:100	19–21. One survivor.†	20
" " "	1	1:1,000	Survived.†	
" " "	1	1:10,000	" †	
" " "vaccinated."*	20	1:100	8–10–10–12–18–18–21–34. Twelve survivors.†	17

* The "vaccinated" mice had received three subcutaneous injections previous to the intraperitoneal or intrastomachal injection.

† All survivors were discarded after 62 days.

protected against a subsequent introduction of the homologous live organisms intraperitoneally or *per os*, and that each dose of "vaccine" seems to confer a similar relative increase in resistance to the peritoneal or gastrointestinal route of infection. Again, given a fixed dose of this culture, it is clear that intraperitoneal injection into normal mice produces an acute type of disease, while the intrastomachal route is followed by a more chronic infection. It would appear, then, that a short interval of time is consumed by the organism in overcoming the

natural defenses of the peritoneal cavity and that a longer period of time is necessarily consumed in overcoming the natural defenses of the gastrointestinal tract, and that this time relationship is retained in immune mice.

Immunization per Os.

The experiments described bring out the nature and the limitations of the immunizing process so far as it is affected by the use of killed cultures injected into the pleural or peritoneal cavity and the subcutaneous tissues. However, the parenteral introduction of the bacilli, whether living or dead, is, as it were, a circumvention of the ordinary or natural process of infection and even perhaps of immunization. Mouse typhoid, so called, is induced by the ingestion of certain paratyphoid-enteritidis strains; and it is to be supposed that in the course of an outbreak of mouse typhoid some animals ingest the cultures, resist infection, and survive. That this is not a hypothesis is shown by the observations of Amoss,[3] by the reported studies of Topley,[4] as well as by our own experiments related above on infection *per os.*

The next step, therefore, in our experimental studies was to attempt immunization directly *per os.* Two general lines were pursued. Certain mice were fed fixed doses of the killed cultures and others were fed very minute amounts of living organisms, in both instances not once only, but over definite periods of time. Mice thus prepared were then tested for resistance by *per os* and intraperitoneal injection of living cultures in lethal doses. Protection was established by both methods, and in contrasting the results of the intrastomachal with the intraperitoneal injections, an attempt was made to determine the occurrence of a possible local gastrointestinal immunity.

Killed Cultures per Os.—The experiments to be described are somewhat intricate, but they are given in detail just because they bring out certain difficulties attending the systematic experimental investigation of mouse typhoid infections.

24 mice were fed daily for 30 days with bread soaked in a broth culture of Bacillus M. T. II, killed by heating to 55°C. for 1 hour. All the animals were

[4] Topley, W. W. C., *J. Hyg.*, 1920–21, xix, 350; *Lancet*, 1919, ii, 1.

living at the end of the feeding period, but during the next 30 days 12 died and at autopsy yielded cultures of Bacillus M. T. II. This latter point will be considered presently. The 12 survivors and 12 controls were then given by stomach tube 1 cc. of a living 1:100, 16 hour broth culture of Strain M. T. II. Of these, 1 "vaccinated" mouse died on the 12th day and yielded at autopsy a positive culture; 7 controls died within 20 days (Table XIII). The 11 survivors were then tested for blood agglutinins and for carriage of Bacillus M. T. II. The blood serum of 5 mice agglutinated the bacillus completely and of 3 mice partially in dilutions of 1:200; 6 of the 11 mice proved fecal carriers of the bacillus.

TABLE XIII.

Per Os Injection of Strain M. T. II into Mice "Vaccinated" per Os with Killed Cultures.

Mice.	No. of mice.	Duration of life in fatal cases.	No. of survivors.
		days	
"Vaccinated."	12	12	11*
Controls.	12	6–7–8–9–12–17–20	5

* "Vaccinated" survivors were used again after 37 days (see Table XIV).

TABLE XIV.

Intraperitoneal Injection of Strain M. T. II into Mice "Vaccinated" per Os with Killed Cultures.

Mice.	No. of mice.	Broth dilution.	Duration of life in fatal cases.	No. of survivors.
			days	
"Vaccinated."	11	1:500		11*
Control.	1	1:100	1	0
"	5	1:500	1–3–3–4–4	0
"	1	1:1,000	3	0
"	1	1:10,000	8	0

* Survivors were discarded after 30 days.

The 11 mice were next injected intraperitoneally with 1 cc. of a 1:500 living broth culture 16 hours old. The control mice were similarly injected with dilutions of 1:100, 1:500, 1:1,000, and 1:10,000. Table XIV presents the result of these tests. While all the control animals succumbed to this active living culture, the treated animals successfully resisted many lethal doses. Not only had the 11 mice become refractory to intrastomachal injections of living cultures which at most converted them into "carriers," but they resisted intraperitoneal injections as well.

The following interpretations of this experiment are offered. The first 12 mice which succumbed to M. T. II infection may have responded to a possible very few living bacilli contained in the "vaccine" (control plates on 3 of the 30 days showed one or two colonies) or may have been infected by the attendant who, in caring for the mice, spread living bacilli from cage to cage. Although this was noted during the epidemiological experiments of Amoss,[2] it may be stated that in our own study great precaution was taken to avoid this complication and that numbers of other normal mice held as controls at the same time and throughout the experiments did not spontaneously develop mouse typhoid. Whether the surviving "vaccinated" mice which resisted the subsequent lethal dose *per os* were protected by the repeated and large doses of killed cultures or the very few possible live organisms which they may have ingested, cannot, from this experiment, be definitely stated; the former supposition, however, is very much more probable.

Living Cultures per Os.—Experimental data as well as observations on the manner of spread of the infection in epidemics of mouse typhoid all indicate that certain mice receive the bacilli of mouse typhoid into the stomach and intestines without succumbing to the disease itself. These facts, taken together with the effect of killed cultures fed *per os* in inducing resistance to infection by living bacilli, led to the employment of minute doses of the living cultures in order to develop this refractory state.

The procedure was as follows: A 14 hour broth culture of Strain M. T. II was fed to 18 mice, so that 3 received 1 cc. each of a 1:1,000 dilution every day, 3 received the same treatment with a 1:10,000 dilution; 3 received the 1:1,000 dilution every 2nd day, 3 the 1:10,000 dilution every 2nd day; 3 the 1:1,000 and 3 the 1:10,000 dilutions every 3rd day. Feedings were discontinued after 34 days. During this period and a subsequent 27 days, all except 7 mice had succumbed to infection with Bacillus M. T. II. The 7 survivors were distributed as follows: 1 from 1:10,000 every day group; 1 from 1:1,000 every 2nd day group; 1 from 1:10,000 every 2nd day group; 2 from 1:1,000 every 3rd day group; and 2 from 1:10,000 every 3rd day group. The 7 survivors, along with 7 control mice, were now given by stomach tube 1 cc. of a 1:100 dilution of Culture M. T. II. Table XV shows the result.

The 6 surviving mice were tested (*a*) for agglutination and (*b*) for carriage of Bacillus M. T. II. The blood serum of 1 mouse agglutinated the bacilli completely in a dilution of 1:200, and 2 of the 6 proved to be fecal carriers. They

were then injected intraperitoneally with 1 cc. of a 1:500 living broth culture 16 hours old. The control mice were similarly infected with dilutions of 1:100, 1:500, 1:1,000, and 1:10,000. Table XVI summarizes the duration of life in experimental and control animals.

TABLE XV.

Per Os Injection of Strain M. T. II into Mice "Vaccinated" per Os with Live Cultures.

Mice.	No. of mice.	Duration of life in fatal cases.	No. of survivors.
		days	
"Vaccinated."	7	7*	6†
Controls.	7	5–6–6–7–9–19–36	0

* This mouse showed no pathological lesions at autopsy. Heart's blood and spleen were sterile.

† Survivors were used again after 38 days (see Table XVI).

TABLE XVI.

Intraperitoneal Injection of Strain M. T. II into Mice "Vaccinated" per Os with Live Cultures.

Mice.	No. of mice.	Broth dilution.	Duration of life in fatal cases.	No. of survivors.
			days	
"Vaccinated."	6	1:500		6*
Control.	1	1:100	1	0
"	5	1:500	1–3–3–4–4	0
"	1	1:1,000	3	0
"	1	1:10,000	8	0

* Survivors were discarded after 30 days.

The results of this experiment are of considerable interest in that they bring out the varying capacities of mice of one age and condition to accommodate to sublethal doses of a given culture of Strain M. T. II introduced into the stomach. The response to wide fluctuations of dose is particularly informing. And the experiment shows unmistakably that the initially and doubtless reinforced refractory mice may come to resist intrastomachal or intraperitoneal doses of the living culture to which all controls succumb.

That protection is afforded certain mice by successive feedings either of heat-killed or living cultures of this paratyphoid-enteritidis strain is indicated by the above experiments. The next tests were devised to bring out the mechanism of this protection—whether, for example, it is a local process confined to the gastrointestinal tract or a general phenomenon operating against the subsequent injection of organisms introduced into the peritoneal cavity as well as into the stomach.

50 mice were tested for blood agglutinins in serum dilutions of 1:20 and 1:200. All were negative. Stool cultures of these mice showed no organisms of the paratyphoid-enteritidis group. "Vaccine" prepared as follows was then added

TABLE XVII.

Intraperitoneal and per Os Injection of Strain M. T. II into Mice "Vaccinated" per Os with Killed Cultures.

Mice.	No. of mice.	Broth dilution.	Duration of life in fatal cases.	No. of survivors.
			days	
Intraperitoneal controls.	4	1:100	1–2–3–3	0
" "	1	1:1,000	8	0
" "	1	1:10,000	9	0
" "	1	1:100,000	7	0
" "vaccinated."	16	1:100	4–4–4–4–4–6–6–6–8–8–9–16	4*
Per os controls.	1	1:10	7	0
" " "	4	1:100	9–10–50	1
" " "vaccinated."	17	1:100	5–6–7–11–12–16–17–25–31–51	7

* Survivors were discarded after 70 days.

daily to the feedings. 10 cc. of a 24 hour broth culture of Strain M. T. II, heated at 55°C. for 2 hours, were added to 40 cc. of milk, soaked up with bread, and divided roughly into ten parts so that each jar of 5 mice received approximately 1 cc. of the original "vaccine." This relatively small dose was continued over a period of 4 weeks. After an interval of 1 week, when it was seen that all the mice were in good condition, agglutination tests were again set up and stool cultures were taken, with negative results.

16 of these mice were then given intraperitoneally 1 cc. of a 1:100 dilution of a 24 hour broth culture of Strain M. T. II; 17 mice received the same dosage *per os*. Controls were injected intraperitoneally and *per os* with doses from 1:10 to 1:100,000. The duration of life in the series is analyzed in Table XVII and Text-fig. 6. Intraperitoneal injection was lethal in a dilution of 1:100,000; the intraperitoneal controls were dead in 3 days. Of the "vaccinated" mice

receiving the intraperitoneal injection, 30 per cent died on the 4th day, 75 per cent were dead on the 16th day, and 25 per cent survived over a period of 10 weeks. The animals receiving live organisms *per os* were somewhat irregular; 80 per cent of the controls succumbed, while only 55 per cent of the "vaccinated" mice died within a period of 10 weeks.

In this experiment we have established, therefore, as a response to the continued ingestion of killed organisms in small doses, a partial protection against live organisms introduced by mouth or by way of the peritoneal cavity, and this protection is in the nature of a general rather than a local immunity.

Finally, to show the general nature of the protection in a more striking manner, immunization was carried out with live organisms.

TABLE XVIII.

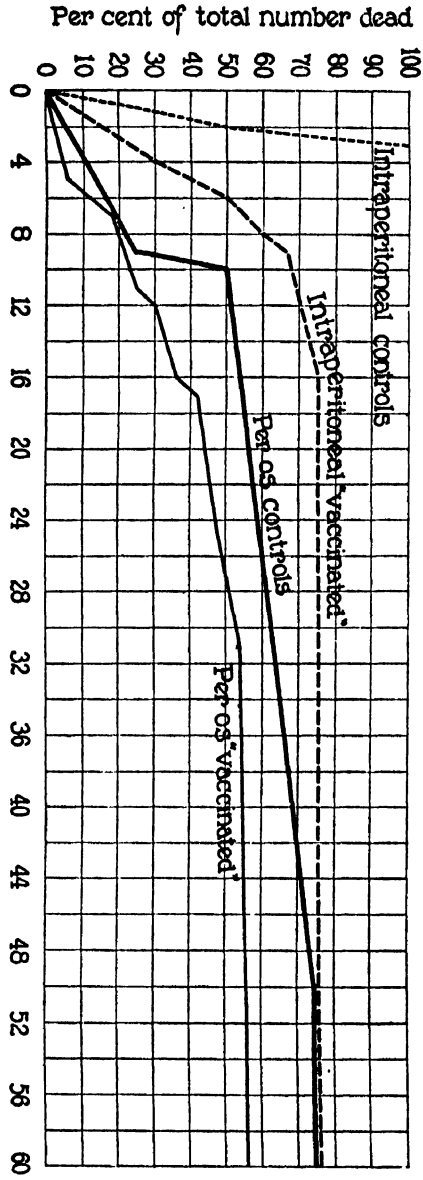
Intraperitoneal Injection of Strain M. T. II into Mice "Vaccinated" per Os with Live Cultures.

Mice.	No. of mice.	Broth dilution.	Duration of life in fatal cases.	No. of survivors.
			days	
Experimental.	18	1:1,000	2–6–12–16	14*
Controls.	1	1:100	1	0
"	7	1:1,000	1–1–1–1–2–2–4	0
"	1	1:10,000	3	0

* Survivors were discarded after 30 days.

25 mice were tested for blood agglutinins for Bacillus M. T. II with serum dilutions of 1:20 and 1:200. None of the mice reacted. Daily feedings of approximately 1 cc. per mouse were now begun with a 16 hour living broth culture of Strain M. T. II diluted 1:10,000 in milk. The total quantities were as follows: 5 mice received 5, 5 received 7, 5 received 9, 5 received 11, and 5 received 13 feedings. Within a 20 day period, 6 of the fed mice succumbed to mouse typhoid infection. The second test for agglutinins was made 13 days after the feedings began and none were found. 23 days later, or 36 days after the experiment began, of 18 surviving mice, 7 showed blood agglutinins in 1:200 dilution. Only 1 mouse was determined to be an M. T. II bacillus carrier. This experiment was terminated by inoculating intraperitoneally the entire 18 survivors of the feeding series along with controls with 1 cc. of a 1:1,000 dilution of an 8 hour living broth culture of Strain M. T. II. Table XVIII shows the result.

While all the controls died, 14 of the fed mice survived. None of the 4 fatalities had previously shown blood agglutinins. It may be



TEXT-FIG. 6. Duration of life of "vaccinated" mice after intraperitoneal and *per os* injections of Strain M. T. II.

seen, therefore, that mice which ingest living cultures of Strain M. T. II and survive may develop a general immunity accompanied in a certain number of cases by blood agglutinins.

DISCUSSION.

As part of a more general study of mouse typhoid infection the experiments in this paper relate to the manner of interaction between the mouse and a native pathogen belonging to the paratyphoid-enteritidis group. The results may be stated in summary about as follows:

Among any considerable number of healthy mice of a given age and size a small number will prove refractory to inoculation with a minimum lethal dose of a mouse strain of *Bacillus pestis caviæ*, no matter into what part of the body the proper injection is made. Minimum lethal dosage is, therefore, a highly relative matter.

Taking the far greater part of the mice chosen for experiment, however, the duration of life and type of infection depend on the dose, the site of injection, and on the individual resistance of the animals as ordinarily present or as artificially produced through immunization. Lesions found at autopsy depend somewhat upon the site of the injection, but chiefly upon the duration of life after the inoculation.

When fixed doses of this organism are injected into the pleura and peritoneum of normal mice, there occurs first an initial period of lag in bacterial growth, during which time—4 to 6 hours—the number of bacilli decreases; subsequently the bacilli multiply rapidly until death occurs, usually within 8 days. This demonstration *in vivo* of bacterial lag parallels in a rough manner the careful work of Penfold,[5] Chesney,[6] and Graham-Smith[7] on bacterial growth *in vitro*.

When mice are "vaccinated" with dead bacilli into the pleural or peritoneal cavity or even subcutaneously, the process of infection is altered. Live bacilli injected into the pleural and peritoneal cavities of such animals rapidly diminish in number and may apparently disappear entirely from the exudate. But usually after remaining at a low level for 2 or 3 days, they gradually begin to increase and continue a slow rise until death ensues, within about 18 days. Accord-

[5] Penfold, W. J., *J. Hyg.*, 1914, xiv, 215.
[6] Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.
[7] Graham-Smith, G. S., *J. Hyg.*, 1920–21, xix, 133.

ing to degree of "vaccination" the subsequent inoculation of live cultures may produce death only after a greater time period or may fail to kill.

Paul Ehrlich was probably the first to demonstrate the possibility of immunization by the gastrointestinal route.[8] By feeding ricin and abrin food cakes to mice, the animals not only developed a tolerance to the feeding of several lethal doses but resisted 400 lethal doses injected subcutaneously. Loeffler,[9] Wolf,[10] Yoshida,[11] and Brückner,[12] by feeding killed or living cultures of certain animal paratyphoid strains to mice, have established a definite immunity against the subsequent feeding in large doses of the homologous live organism. The controversy throughout this work as to whether the induced gastrointestinal immunity is local or general has been renewed by the recent work of Besredka with dysentery and paratyphoid bacilli on mice and rabbits.[13] He offers the somewhat startling hypothesis that not only is the gastrointestinal immunity entirely of a local nature, but that any mode of vaccination is followed by only one type of immunity—local: "Tout comme l'immunité naturelle, l'immunité artificielle vis-à-vis des virus typhique et paratyphique repose sur celle de la paroi intestinale: elle est d'essence locale."

Throughout our work there has been no evidence of acquired local immunity as opposed to a condition of general immunity. The feeding of killed or living cultures of a mouse typhoid strain protects the mice against lethal doses of living bacilli injected *per os* or intraperitoneally. And when killed bacilli are injected into the pleura or peritoneum, those cavities are rendered no more resistant to the introduction of the living bacilli than when the "vaccination" is made subcutaneously. It seems, therefore, that protective principles following the introduction of killed or living bacilli into any part of the body operate against the later injection of living organisms, regardless of their portal of entry.

[8] Ehrlich, P., *Deutsch. med. Woch.*, 1891, xvii, 976, 1218.

[9] Loeffler, F., *Gdnkschr. verstorb. Generalstabsarzt Armee . . . . v. Leuthold*, 1906, i, 249.

[10] Wolf, K., *Münch. med. Woch.*, 1908, lv, 270.

[11] Yoshida, E., *Arch. Hyg.*, 1909, lxix, 21.

[12] Brückner, G., *Z. Immunitätsforsch., Orig.*, 1910–11, viii, 439.

[13] Besredka, A., and Basseches, S., *Ann. Inst. Pasteur*, 1918, xxxii, 193. Besredka, A., *Ann. Inst. Pasteur*, 1919, xxxiii, 301, 557, 882.

A word should be said concerning experimental infection *per os*, the normal portal of entry. Whereas earlier writers have experienced difficulty in producing the disease in this manner, we have found it possible, within certain limits, to infect regularly. These limits, however, are subject to such fluctuations that results must be interpreted with caution. In these experiments two facts must be remembered: first, that all tests were conducted in large battery jars containing either 3 or 6 mice and that this constant contact factor must have raised the per cent of mortality in controls as well as experimental animals; secondly, that only wide deviations in mortality curves were considered as significant.

SUMMARY.

If live cultures of a mouse strain of *Bacillus pestis caviæ* are injected intrapleurally or intraperitoneally into normal mice, there occurs an initial lag in the rate of bacterial multiplication lasting a few hours, followed by a rapid and continued acceleration of growth until the death of the animal.

If live cultures of this organism are given *per os* to normal mice, there occurs an incubation period of 5 to 6 days, after which the animal usually develops symptoms of disease and succumbs. A small percentage of mice, however, proves refractory to infection by this route.

If live cultures of this organism are injected intrapleurally or intraperitoneally into mice previously "vaccinated" intrapleurally or intraperitoneally, they are partially destroyed and held in check by the protective mechanisms of the animal body for 2 or 3 days. Subsequently the rate of bacterial multiplication increases gradually until the death of the animal. The partial protection following this type of "vaccination" is entirely of a general nature; no evidence of a local immunity has been obtained.

Mice given one, two, or three subcutaneous doses of "vaccine" show a similar relative increase in resistance to the subsequent intraperitoneal or *per os* injection of live organisms.

Feeding mice live or killed cultures of this organism induces a definite protection against subsequent intrastomachal and intraperitoneal injections of live organisms. The immunity developed in this way is also of a general as opposed to a local nature.

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IDENTIFICATION OF A PARATYPHOID-ENTERITIDIS STRAIN ASSOCIATED WITH EPIZOOTICS OF MOUSE TYPHOID.*

BY LESLIE T. WEBSTER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, April 5, 1922.)

In other papers of this series, Amoss[1] and Lynch[2] have described epizootics of mouse typhoid associated with a bacillus of the paratyphoid-enteritidis group, and with this same organism, designated as Strain M. T. II, the author has studied certain corollary protection and immunity phenomena.[3] It seems important, therefore, to classify definitely this M. T. II strain and to relate it as far as possible to other similar pathogens.

B. typhi murium, isolated by Loeffler[4] from an epizootic of laboratory mice and related to the colon-typhoid group, was considered by him to be the causative microbe of mouse typhoid. In France this etiological rôle was assigned to a similar organism, Danysz' bacillus, recovered from a plague of field mice.[5] Later serological studies of Bainbridge[6] on the paratyphoid and food poisoning bacilli indicate that Danysz' bacillus is identical with *B. enteritidis* Gärtner and that *B. typhi murium* does not exist as an entity since some strains correspond to

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* In a communication to the Society for Experimental Biology and Medicine (Webster, L. T., *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 71) this organism was designated as *B. enteritidis* (*murium*).

[1] Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25.

[2] Lynch, C. J., *J. Exp. Med.*, 1922, xxxvi, 15.

[3] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 71.

[4] Loeffler, F., *Centr. Bakt.*, 1892, xi, 129.

[5] Danysz, J., *Ann. Inst. Pasteur*, 1900, xiv, 193.

[6] Bainbridge, F. A., *J. Path. and Bact.*, 1909, xiii, 443.

B. enteritidis and others to *B. aertrycke.*[7] Krumwiede[8] has found that strains of so called *B. typhi murium* belong either to the enteritidis group or to a class of paratyphoid-like strains of rodent origin (*B. pestis caviæ.*)[9] Finally, throughout Topley's study of an experimental mouse epizootic induced by the feeding of *B. enteritidis*, his autopsy protocols note the recovery of *B. enteritidis* and *B. aertrycke.*[7,10] Apparently, then, *B. typhi murium* has no significance as an entity; Danysz' bacillus is synonymous with *B. enteritidis* Gärtner; and the microbes usually associated with mouse typhoid are *B. enteritidis* Gärtner, *B. aertrycke*, and *B. pestis caviæ* Smith.

Morphological and Cultural Characteristics.

Strain M. T. II is a Gram-negative, motile, non-sporulating bacillus. Agar colonies are thin, bluish, and somewhat translucent, with irregular edges. Dextrose, levulose, maltose, mannitol, xylose, arabinose, rhamnose, and inosite are fermented. Gas is formed, milk is not coagulated, indole is not produced, lead acetate medium is blackened.

These characteristics place the organism in the paratyphoid-enteritidis group. The fermentation of arabinose and the reaction in lead acetate medium would tend to exclude *Bacillus suipestifer*, while the vigorous production of acid in inosite eliminates *Bacillus enteritidis* and favors the paratyphoid group. However, it is necessary to correlate these observations with the more accurate information derived from serological tests.

Serological Studies.

An immune rabbit serum was prepared from Strain M. T. II. Stock cultures of the paratyphoid-enteritidis group were seeded into plain broth, pH 7.4, incubated for 24 hours, and mixed with equal parts of 0.2 per cent formalin in 0.85 per cent salt solution. Agglutination tubes were set up to contain one part of serum dilution to nine

[7] Topley, Weir, and Wilson (Topley, W. W. C., Weir, H. B., and Wilson, G. S., *J. Hyg.*, 1921, xx, 241) make the following statement in regard to paratyphoid bacilli of animal origin: "It is probable that *B. aertrycke* belongs to this subgroup, and also those *B. suipestifer* strains of German origin which were studied by Bainbridge and O'Brien (1911). The bacilli isolated from mice dying during our own experiments, and hitherto referred to as *B. suipestifer*, have all the characteristics of this subgroup and should be placed in it."

[8] Krumwiede, C., Jr., Valentine, E., and Kohn, L. A., *J. Med. Research*, 1918–19, xxxix, 449.

[9] Wherry, W. B., *J. Infect. Dis.*, 1908, v, 519.

[10] Topley, W. W. C., *J. Hyg.*, 1920–21, xix, 350.

parts of formalinized antigen. The several strains employed may be described as follows:

M. T. II. Unknown strain for identification.
B. pestis caviæ, No. 146. (*Ferry 63*).[8]
320 mutton type. "*Aertrycke*" strain from Schütze;[11] called by him "Mutton type Calf 6."
B. choleræ suis, No. 350 (*Smith*). Isolated by TenBrocck in 1919.
B. paratyphosus B. No. 178 Army strain.
313 Stanley. "*Aertrycke*" strain from Schütze; Hutchins.[11]
315 G. "*Aertrycke*" strain from Schütze; monkey.[11]
316 Reading. "*Aertrycke*" strain from Schütze; water supply.[11]
317 Newport. "*Aertrycke*" strain from Schütze; Fentry strain; isolated by Perry.[11]
B. enteritidis, No. 47. Gärtner.
B. enteritidis, No. 273. McWeeny strain; from Jordan.
B. abortus equi, 215 Meyer No. 7. Kentucky.
B. paratyphosus A. Army strain.

From Table I it may be seen that while Serum M. T. II fails to agglutinate *Bacillus enteritidis*, *Bacillus abortus equi*, and *Bacillus paratyphosus* A, and while it reacts only slightly with the Army strain of *Bacillus paratyphosus* B, *Bacillus choleræ suis*, and the Stanley, G, Reading, and Newport *aertrycke* strains of Schütze, it does agglutinate the "mutton" strain of Schütze and *Bacillus pestis caviæ* to a titer approximating that of the homologous strain.

To emphasize this relationship, sera of the various type strains were set up against Strain M. T. II (Table II).

This cross-agglutination eliminates the Army Para B, *Bacillus choleræ suis*, and the Stanley, G, Reading, and Newport strains. "Mutton" serum, however, agglutinates Strain M. T. II to nearly as high a titer as the homologous strain and *Bacillus pestis caviæ* serum agglutinates Strain M. T. II and homologous strain to equal titer.

Final evidence relating Strain M. T. II to *Bacillus pestis caviæ* and the "mutton" strain was obtained by absorption tests.

Some quantitative measurement of absorbing antigen is necessary for accurate interpretation of absorption phenomena. In these tests,

[11] Schütze, H., *Lancet*, 1920, i, 93.

therefore, a constant ratio between each serum volume and the volume of the absorbing strain was determined by measuring the volume of packed cell antigen and then adding the calculated amount of serum diluted 1:10. For the low titer "mutton" serum this ratio was 1:4; for the *Bacillus pestis caviæ* serum a 1:2 ratio was employed, while in the case of Serum M. T. II, it was necessary to absorb twice with a ratio of packed cells to serum of 1:3. For controls each absorption

TABLE I.

Agglutination with Serum M. T. II against Various Strains.

Strain.	Serum M. T. II dilution.							
	1:100	1:1,000	1:5,000	1:7,000	1:9,000	1:10,000	1:20,000	1:50,000
M. T. II.	++	++	++	++	++	++	++	−
B. pestis caviæ, No. 146.	++	+1	+1	+1	+1	+1	+	−
320 mutton.	++	++	++	+1	+1	+1	+	−
B. choleræ suis, No. 350 (Smith).	+1	+1	−	−	−	−	−	−
Para B Army.	+1	+1	−	−	−	−	−	−
313 Stanley.	+1	+	−	−	−	−	−	−
315 G.	+1	+1	−	−	−	−	−	−
316 Reading.	+1	+1	−	−	−	−	−	−
317 Newport.	+	+	−	−	−	−	−	−
B. enteritidis, No. 47.	−	−	−	−	−	−	−	−
" " " 273.	−	−	−	−	−	−	−	−
" *abortus equi*.	−	−	−	−	−	−	−	−
Para A Army.	−	−	−	−	−	−	−	−

++ indicates complete agglutination; +1, marked agglutination; +, slight agglutination; 1, trace of agglutination.

test was paralleled by agglutination of unabsorbed serum with homologous and heterologous strains and by absorption and subsequent agglutination with the homologous strain. The results are presented in Tables III to VI.

Table III shows that Strain M. T. II completely absorbed its own agglutinins from "mutton" serum and 98 per cent of the homologous agglutinins. Conversely (Table IV), the "mutton" strain, after one absorption, removed 90 per cent of its own agglutinins from Serum M. T. II and 75 per cent of the homologous agglutinins, and, after

two absorptions, the "mutton" strain removed all of its own agglutinins and 93 per cent of the homologous agglutinins.

It is probable then, that Strain M. T. II and the "mutton" strain are antigenically very similar but not quite identical.

TABLE II.

Direct Agglutination with Various Sera against Strain M. T. II.

Serum.	Strain.	Serum dilution.											
		1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:102,400	Control.
B. pestis caviæ.	*B. pestis caviæ.*	++	++	++	++	++	++	+	1	−	−	−	−
	M. T. II.	++	++	++	++	++	++	+	1	−	−	−	−
"Mutton."	"Mutton."	++	++	++	++	++	+1	1	1	−	−	−	−
	M. T. II.	++	++	++	++	++	1	−	−	−	−	−	−
B. choleræ suis, No. 350.	*B. choleræ suis*, No. 350.	++	++	++	++	++	++	+1	+	+	1	−	−
	M. T. II.	−	−	−	−	−	−	−	−	−	−	−	−
B. paratyphosus B, No. 178.	*B. paratyphosus* B.	++	++	++	++	++	++	+1	+	+	1	−	−
	M. T. II.	−	−	−	−	−	−	−	−	−	−	−	−
313 Stanley.	313 Stanley.	++	++	++	++	++	+	1	−	−	−	−	−
	M. T. II.	−	−	−	−	−	−	−	−	−	−	−	−
315 G.	315 G.	++	++	++	++	++	++	+	+	1	−	−	−
	M. T. II.	−	−	−	−	−	−	−	−	−	−	−	−
316 Reading.	316 Reading.	++	++	++	++	++	++	+	−	−	−	−	−
	M. T. II.	−	−	−	−	−	−	−	−	−	−	−	−
317 Newport.	317 Newport.	++	++	++	++	++	+	−	−	−	−	−	−
	M. T. II.	−	−	−	−	−	−	−	−	−	−	−	−

Table V shows that Strain M. T. II absorbed 97 per cent of its own agglutinins from *Bacillus pestis caviæ* serum and 97 per cent of the homologous agglutinins. Conversely (Table VI), the *Bacillus pestis caviæ* strain after one absorption removed 98 per cent of its own agglutinins from Serum M. T. II and 98 per cent of the ho-

TABLE III.

"Mutton" Serum.

Strain.	Unabsorbed serum.											Serum absorbed by "mutton" strain.											Serum absorbed by Strain M. T. II.										
	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:102,400	Control.	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200
"Mutton."	++	++	++	++	++	+	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	1	—	—	—	—	—	—	—	—
M. T. II	++	++	++	++	+	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

TABLE IV.

Serum M. T. II.

Strain.	Unabsorbed serum.												Single absorption by Strain M. T. II.											Single absorption by "mutton" strain.										
	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:102,400	Control.	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:102,400	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:102,400
M. T. II..	++	++	++	++	++	++	++	++	+1	1	—	—	++	++	++	+1	+	1	—	—	—	—	—	—	++	++	++	++	++	++	++	+1	+	—	—	—
"Mutton."	++	++	++	++	++	++	++	+1	+	1	—	—													++	++	++	++	+1	+1	+	—	—	—	—	—

Strain.	Double absorption by Strain M. T. II.											Double absorption by "mutton" strain.										
	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200
M. T. II	—	—	—	—	—	—	—	—	—	—	—	+1	+1	+1	+1	+1	+1	—	—	—	—	—
"Mutton."												—	—	—	—	—	—	—	—	—	—	—

TABLE V.

B. pestis caviæ Serum.

Strain.	Unabsorbed serum.												Serum absorbed by *B. pestis caviæ*.												Serum absorbed by Strain M. T. II.											
	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:102,400	Control.	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200		1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	
B. pestis caviæ...	++	++	++	++	++	++	+1	+	1	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	—	—	—	—	—	—	—
M. T. II	++	++	++	++	++	++	++	+1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	—	—	—	—	—	—	—

TABLE VI.

Serum M. T. II.

Strain.	Unabsorbed serum.												Single absorption by Strain M. T. II.												Single absorption by *B. pestis caviæ*.											
	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:102,400	Control.	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:102,400	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:102,400
M. T. II..	++	++	++	++	++	++	++	++	+1	1	—	—	++	++	++	+1	+	1	—	—	—	—	—	—	++	++	++	++	+	1	—	—	—	—	—	—
B. pestis... *caviæ*....	++	++	++	++	++	++	++	+1	+1	1	—	—		+1	+	+	+	—	—	—	—	—	—	—	++	++	++	+1	+	—	—	—	—	—	—	—

Strain.	Double absorption by Strain M. T. II.												Double absorption by *B. pestis caviæ*.											
	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200		1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	
M. T. II.....	—	—	—	—	—	—	—	—	—	—	—		—	—	—	—	—	—	—	—	—	—	—	
B. pestis caviæ.	—	—	—	—	—	—	—	—	—	—	—		—	—	—	—	—	—	—	—	—	—	—	

mologous agglutinins, and, after two absorptions, the *Bacillus pestis caviæ* strain removed all of its own agglutinins and all of the homologous agglutinins from Serum M. T. II.

Clearly, then, Strain M. T. II and the type strain *Bacillus pestis caviæ* are antigenically identical.

DISCUSSION.

The "mutton" group forms the chief division in Schütze's serological study of the paratyphoid *aertrycke* types.[11] He says: "The Mutton group claims over 50 per cent of the strains, and would appear to be the most important; it includes strains from various countries and from animal as well as human epidemics."

The *Bacillus pestis caviæ* group, "a distinct paratyphoid type or group of bacilli, is encountered therefore in spontaneous infections in laboratory animals, especially rodents" according to Krumwiede.[8] Thirteen guinea pigs, two mice, one cat, and one rabbit strains were found by him to be antigenically similar.

Strain M. T. II is apparently similar to the type "mutton" strain of Schütze and is identical with the type *Bacillus pestis caviæ* strain.[12]

To have closely related this mouse typhoid strain to other paratyphoid types is to emphasize once again the ubiquity of the paratyphoid group and the possibility that the various strains found in mice, rats, guinea pigs, sheep, and doubtless in other domestic animals, active, capable of producing epizootics, may likewise be human pathogens with greater or less degree of virulence. Precise information concerning the underlying principles of mouse typhoid infection and epizootics should, therefore, be of great service in the interpretation of similar phenomena of man.

CONCLUSIONS.

A bacillus of the paratyphoid-enteritidis group associated with epizootics among laboratory mice has been identified with *Bacillus pestis caviæ* Smith which produces similar affections in guinea pigs and has been very closely related to the type "mutton" *aertrycke* strain of Schütze.

[12] Unpublished studies of Krumwiede and Cooper have already demonstrated a close relation between the *B. pestis caviæ* group and the "mutton" types of Schütze.

This identification is based upon the cultural reactions of the organism, direct and cross-agglutinations, and similar absorptive capacities of the unknown and type strains.

The author wishes to thank Dr. Charles Krumwiede and Miss Georgia Cooper for the type strains and type antisera and for assistance especially as regards the technical details of the absorption method which they employ.

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IMMUNOLOGICAL DISTINCTIONS OF TWO STRAINS OF THE MOUSE TYPHOID GROUP ISOLATED DURING TWO SPONTANEOUS OUTBREAKS AMONG THE SAME STOCK.

BY HAROLD L. AMOSS, M.D., AND PETER P. HASELBAUER.
(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, April 6, 1922.)

In the cancer breeding station maintained at The Rockefeller Institute there occurred, during 2½ years, two separate outbreaks of mouse typhoid among the 2,500 to 4,000 mice. The first of these appeared in the autumn of 1918 and the second exactly 2 years later. They are described by Lynch[1] in a separate paper of this series.

From the spleens of mice dying in the first outbreak there were isolated several strains which belonged to the paratyphoid-enteritidis group. One of them, Mouse Typhoid I, was selected for use in inciting an experimental epidemic, but before starting the experiments, this strain was passed through four mice *per os* in order to assure its pathogenic power. The strain, recovered from the fourth mouse, was used in a series of artificially produced epidemics among mice for more than 2 years. Meanwhile, antiserum had been prepared against Mouse Typhoid I, and while active in high dilution against the homologous strain, it did not agglutinate the unidentified strain. It was evident that the two strains, both belonging to the paratyphoid-enteritidis group as determined by fermentation tests, were not identical. Antiserum against the new strain was prepared, but it did not agglutinate Mouse Typhoid I. This serum, however, did agglutinate the strains isolated from the spleens of mice dying in the experimental epidemic, and the inference is that the unidentified strain (now called Mouse Typhoid II) was the organism concerned in the epidemic, for no members of Mouse Typhoid I type were obtained.

In the preliminary feeding experiment to which reference has been made, Mouse Typhoid I was employed, but Mouse Typhoid II was recovered from the spleen of the fourth mouse in the series. The

[1] Lynch, C. J., *J. Exp. Med.*, 1922, xxxvi, 15.

probable explanation is that one of the mice used in the experiment was already infected with Mouse Typhoid II. This view is strengthened by the fact that occasionally among the stock from which these mice were taken deaths occurred and from the spleens only Mouse Typhoid II was isolated. Moreover, in the second epidemic among the cancer mice occurring in the autumn of 1920, Mouse Typhoid II was regularly obtained.

EXPERIMENTAL.

Agglutination Tests.

Monovalent sera were prepared by immunizing rabbits against Mouse Typhoid I, Mouse Typhoid II, and Paratyphoid B Rowland.[2]

TABLE I.

Agglutination of Various Strains of the Paratyphoid-Enteritidis Group in Three Monovalent Antisera.

Antigen.	Highest dilution showing complete agglutination. Serum.		
	Mouse Typhoid I.	Mouse Typhoid II.	Paratyphoid B Rowland.
Mouse Typhoid I	1:2,000	0	0
" " II	0	1:1,500	1:800
Paratyphoid B Rowland (Army Medical School)	0	1:800	1:1,200
Paratyphoid B Black (Army Medical School).	0	1:1,000	1:1,200
" " Kendall I	0	1:1,000	1:4,000
" " " II	0	1:1,000	1:5,000
Enteritidis—Kendall	1:2,000	0	0
" 18 (American Museum of Natural History)	0	0	0
Enteritidis 273 Gärtner	1:800	0	0
Animal typhoid—Smith.			
Mouse 2. Rat. Guinea pig. Dog 1.	>1:400*	0	0
Calf.	0	>1:400*	0

* Higher dilution not made.

[2] Paratyphoid B Rowland is the strain used by the Army Medical School in the preparation of the triple typhoid vaccine.

The results of the agglutination tests with these strains and enteritidis strains and other paratyphoid B strains[3] are shown in Table I.

It is evident that Mouse Typhoid II is closely allied antigenically to the human paratyphoid B strains tested, while Mouse Typhoid I is totally different. Mouse Typhoid I is related to the enteritidis strains Kendall and Gärtner 273 but not to the culture obtained from the American Museum of Natural History.

Animal Strains.

The following animal typhoid strains[4] were roughly classified by agglutination in Mouse Typhoid I and Mouse Typhoid II serum in a dilution of 1:400, as follows: Mouse 2, rat, guinea pig, and Dog 1 strains were completely agglutinated by Mouse Typhoid I serum in 1:400 and not by Mouse Typhoid II serum; while the calf strain was completely agglutinated by Mouse Typhoid II serum and not by Mouse Typhoid I serum.

Absorption Tests.

Mouse Typhoid II and Para B Sera.

Antigen for absorption was prepared by washing 16 hour growths from slant agar pH 7.4 with isotonic salt solution, filtering through cotton wool, and standardizing in a turbidimeter. The absorptions with the same amount of antigen in every case were made four times during 3 hours at 37°C. in dilutions 1:10, 1:20, 1:25, and 1:50 respectively. The results of agglutination tests on the Mouse Typhoid II and Para B sera after absorption are given in Table II.

It appears from these tables that Mouse Typhoid II and Para B are not identical. Para B Rowland and Black strains absorb agglutinins against themselves from Mouse Typhoid II serum, but leave some Mouse Typhoid II agglutinins. Para B serum is absorbed more slowly even by the homologous strain. The difference between Mouse Typhoid II and Para B Rowland and Black is also

[3] Strains of paratyphoid B and enteritidis were obtained from the Army Medical School, Dr. A. I. Kendall, and the American Museum of Natural History.

[4] These strains were furnished by Dr. Theobald Smith.

TABLE II.

Agglutination after Absorption.

Test strain.	Before absorption.	Absorbing Strain.					
		Mouse Typhoid II.	Para B Rowland.	Para B Black.	Para B Kendall I.*	Para B Kendall II.*	Mouse Typhoid I.
Mouse Typhoid II serum.							
Mouse Typhoid II	1:1,500	0	++1:400	++1:500	+1:200 ++1:100	+1:300 +1:200	+++1:1,500
Para B Rowland	1:800	0	0	0	+1:200 ++1:100	0	—
" " Black	1:1,000	0	0	0	+1:200 ++1:100	0	—
" " Kendall I.	1:1,000	++1:100	++1:200	++1:100, 1:200	++1:400 +++1:300	++1:400 +++1:300	—
" " " II	1:1,000	++1:100	++1:200	+++1:100	++1:400 +++1:300	++1:200	—
Calf typhoid	1:400†	0	—	—	—	—	—
Para B Rowland serum. Regular technique four times in 3 hrs.							
Mouse Typhoid II	1:800	+1:100	+1:100	+1:100	+++1:500	+++1:500	—
Para B Rowland	1:1,200	++1:400 +++1:100	+1:300 +1:100	++1:100	+++1:500	+++1:800	—
" " Black	1:1,200	++1:300 +++1:100	++1:300 ++1:100	++1:200 ++1:100	+++1:500	+++1:800	—
" " Kendall I	1:4,000	+++1:1,000	+++1:500	+++1:500	+++1:1,000 ++1:2,000	+++1:2,000	—
" " " II	1:5,000	+++1:2,000	+++1:800	+++1:500	+1:2,000 ++1:1,000	+++1:2,000	—

Para B Rowland serum. Regular technique eight times in 16 hrs.

Mouse Typhoid II	1:800	0	0	0	0	0	—
Para B Rowland	1:1,200	+1:300 ++1:100	0	0	0	0	—
" " Black	1:1,200	+1:300 ++1:100	0	0	0	0	—
" " Kendall I	1:4,000	++1:400 +++1:300	+1:100	+1:200 +1:100	0	0	—
" " " II	1:5,000	++1:400 +++1:300	+1:200	+1:200 ++1:100	0	0	—

+++ indicates complete agglutination, clear above; ++, marked clumping, turbid above; +, definite clumping; 0, no agglutination; —, test not made.

* Absorbed five times.

† Higher dilutions not made.

shown after absorption eight times over 16 hours at 37°C., when Mouse Typhoid II removes all agglutinins reacting against it but leaves agglutinins for Para B strains.

Para B Kendall I and II are still slower in absorbing and are not identical with Para B Rowland.

Mouse Typhoid I has no power of absorption in Mouse Typhoid II serum.

Mouse Typhoid I Serum.

By direct agglutination Mouse Typhoid I seems to be related to four animal typhoid strains and also to Enteritidis Kendall and

TABLE III.

Agglutination after Absorption.

Test strain.	Before absorption.	Absorbing strains.			
		Mouse Typhoid I.	Enteritidis 18.	Enteritidis Kendall.	Enteritidis 273.
Mouse Typhoid I.....	1:2,000	0	+1:1,000 +++1:500	+1:800 +++1:400	+1:1,000 ++1:200
Enteritidis 18.....	0	0	0	0	
" Kendall.....	1:2,000	0	+1:1,000 +++1:400	0	—
" 273.....	1:800	0	—	—	0
Mouse Typhoid 2 (Smith)..	>1:400	0	—	—	0
Rat typhoid (Smith).....	>1:400	0	—	—	0
Guinea pig typhoid (Smith)	>1:400	0	—	—	+1:300
Dog Typhoid 1 (Smith)....	>1:400	0	—	—	+1:800

Enteritidis 273 Gärtner, but the latter are differentiated by absorption with the method already described.

Table III shows that Mouse Typhoid I removes all agglutinins from Mouse Typhoid I serum, but Enteritidis Kendall and 273 absorb all agglutinins against themselves from Mouse Typhoid I serum, but not all which are active against Mouse Typhoid I. The fact that Enteritidis 18, though not agglutinated in Mouse Typhoid I serum, apparently absorbs some of the agglutinins, is perhaps an example of non-specific or physical adsorption.

SUMMARY.

Two strains of the paratyphoid B-enteritidis group causing separate epidemics of mouse typhoid among 2,500 to 4,000 cancer breeding mice are found to be antigenically different. Mouse Typhoid I, isolated from the first outbreak, is related but not identical with two strains of enteritidis, while Mouse Typhoid II is related to but not identical with the human paratyphoid B strains.

In a separate paper in this series, Webster[5] has identified Mouse Typhoid II strain with *Bacillus pestis caviæ* Smith and has suggested its close relation to the *Bacillus aertrycke* (mutton) group of Schütze.

[5] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 97.

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THE ORGANOTROPIC, BACTERIOTROPIC, AND LEUCOCYTOTROPIC ACTIONS OF CERTAIN ORGANIC CHEMICALS.

BY LLOYD D. FELTON, M.D., AND KATHARINE M. DOUGHERTY.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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Present knowledge of the manner by which chemicals influence the mechanism of infection and resistance is limited. Investigations in chemotherapy hitherto have been concerned primarily with certain organotropic and bacteriotropic activities,—a search for a chemical of a monotropic character. The influence of definite chemical entities both upon the factors vital to the integrity of the animal against an invading microorganism, and such life processes as are perhaps not essential to actual defense against bacterial disease, has not been thoroughly investigated. The work of Ehrlich, Morgenroth, Browning, and others has demonstrated that it is possible to find chemicals with less of organotropic than of bacteriotropic activity. Frequently, however, when such substances have been used against an experimental infection, the treated animal succumbed as though no drug had been given, or in a shorter time than the controls. In other words, the drug, in doses not fatal under normal conditions, apparently acts upon defensive complexes necessary to the animal in coping with an infection, having a greater influence upon this activity than upon the pathogenic microorganism. Just what the force or combination of forces is that maintains a state of stable resistance in the animal toward microorganisms can only be conjectured. Whatever other elements enter into this biological reaction, obviously the phagocyte has some share. Because of this, as evidenced in the increase of the activity of the leucocyte during the course of many infectious diseases, it was decided to study the influence of certain chemicals, not alone in respect to organotropism and to bacteriotropism, but as to leucocyto-

tropism as well. The work was undertaken in the hope that information might be gained which would enable a more intelligent choice of chemicals for chemotherapeutic purposes, and might lead to the finding of a delicate indicator by which to judge the relationship between chemical constitution and the activity exhibited by the body against an invading organism.

We wish to report the toxicity for mice (organotropism), the bactericidal action on *Staphylococcus aureus* (bacteriotropism), and the antiphagocytic influence (leucocytotropism), of certain members of seven groups of chemicals—triphenylmethane leuco bases, triphenylmethane dyes, acridines, safranines, phenazines, quinones, and cinchonas.

The work was done while in collaboration with Dr. Jacobs and Dr. Heidelberger in the study of chemotherapy.[1] The manner in which the chemicals here reported influence an experimental pneumococcus infection of mice will appear in a later publication.

The confusion that exists in the literature on phagocytosis seems to be due for the most part to variations in technique. Even in controlled phagocytic studies frequent anomalies appear, unavoidable when dealing with so delicate an indicator as the leucocyte. If, then, instead of considering results from one such experiment or series of experiments, we attempt to compare those from work done under widely differing conditions, definite conclusions are difficult to reach.

The findings of Hamburger,[2] Kolmer,[3] Manwaring and Ruh,[4] Grünspan,[5] and Smith [6] on the influence of quinine on phagocytosis can hardly be compared. Hamburger[7] obtained leucocytes from defibrinated horse blood and suspended them in horse serum. To this suspension he added the drug and incubated the mixture, thus affording opportunity for interaction between quinine and leucocytes

[1] Felton, L. D., and Dougherty, K. M., *J. Exp. Med.*, 1922, xxxv, 761.

[2] Hamburger, H. J., *Centr. Bakt., Ite Abt., Ref.*, 1913, lvii, 105. Hamburger, H. J., and Hekma, E., *Biochem. Z.*, 1908, ix, 512.

[3] Kolmer, J. A., Solis-Cohen, S., and Steinfield, E., *J. Infect. Dis.*, 1917, xx, 333.

[4] Manwaring, W. H., and Ruh, H. O., *J. Exp. Med.*, 1907, ix, 473.

[5] Grünspan, T., *Centr. Bakt., Ite Abt., Orig.*, 1909, xlviii, 444.

[6] Smith, H. L., *Lancet*, 1910, ii, 1342.

[7] Hamburger, H. J., and Hekma, E., *Biochem. Z.*, 1907, iii, 88.

before the charcoal was added. Kolmer,[3] on the other hand, used washed leucocytes procured from the peritoneal cavity of rabbits after an injection of aleuronat, and these came into contact with the quinine only after it had been incubated an hour with a heavy suspension of living Type I pneumococci. Thus in the former case, the quinine acted primarily on the leucocyte and secondarily on the substance to be phagocytosed, while in the latter, the primary action was on the pneumococcus. As might be expected, their results contradict each other, Hamburger[2] claiming an inhibition in phagocytic activity with a dilution of quinine 1:1,000, and Kolmer[3] claiming a stimulation with the same strength of the drug. Manwaring and Ruh,[4] using still another technique, found a 20 per cent stimulation from quinine in as low a dilution as 1:200. In this instance whole defibrinated blood was used with a streptococcus which had been suspended in 0.85 per cent NaCl and sterilized at 100° C. Grünspan[5] also places the optimum concentration for this drug at 1:200. Smith[6] gives 1:7,500 as the lowest dilution of quinine stimulating phagocytosis. He used washed human corpuscles, human serum, and living *B. coli*, sealing them in a Wright tube and incubating in an opsonizer. The fact that the chamber was sealed may have exerted an influence on the process.

Methods.

Although present knowledge relating to phagocytosis does not make it possible to employ a method which will give uniformly constant results, by testing the chemicals in groups, each group on the same day, with the same leucocytes, staphylococci, and guinea pig serum, we have endeavored to render the conditions of our experiments constant.

The following three methods for determining the degree of phagocytosis were tried.

1. 0.5 cc. of leucocytic suspension + 0.5 cc. of culture + 0.5 cc. of guinea pig serum 1:10 + 0.5 cc. of chemical were mixed in a 12 mm. tube and incubated for 15 minutes in a water bath at 37.5°C.

2. 0.5 cc. of leucocytic suspension + 0.5 cc. of chemical were mixed and incubated as above for 10 minutes. To this were then added 0.5 cc. of culture and 0.5 cc. of guinea pig serum 1:10, the mixture was shaken, and the tube was reincubated for 10 minutes in a water bath at 37.5°C.

3. 0.5 cc. of leucocytes suspended in undiluted guinea pig serum + 0.5 cc. of culture + 0.5 cc. of chemical were incubated for 15 minutes in a water bath at 37.5°C.

The last was the method finally adopted, since the simultaneous incubation of all constituents mixed together seemed more nearly to reproduce actual conditions of infection in the animal.

The leucocytes were procured from the peritoneal cavity of a guinea pig 15 hours after an intraperitoneal injection of a solution composed of 3 per cent aleuronat, 6 per cent starch, in 0.85 per cent NaCl, by washing out the exudate with sterile 0.5 per cent citrate in 0.85 per cent NaCl. The cells were washed three times in sterile citrate solution before being used. Opsonin and complement were furnished by fresh guinea pig serum and the organism employed as the indicator of leucocytic activity was an 18 hour broth culture of *Staphylococcus aureus*.

Smears were made after incubation, and the preparations stained by Cross'[8] method. The staphylococci contained in 100 cells were counted and the counts averaged. The result of each dilution of the chemical is shown in Tables I to VIII, the dilution quoted being calculated after the addition of all the components. Uniform dilutions of the different chemicals could not be employed, due to the varying solubilities of the drugs. Control counts were made from tubes containing leucocytes, serum, and organisms, but no chemical.

The toxicity given is the largest, non-fatal intraperitoneal dose in milligrams for 18 gm. mice.

The bactericidal action of the drugs was determined in whole blood with a 2 hour incubation period, by means of a technique described in a previous paper.[1]

Triphenylmethane Leuco Bases.

As a group, the triphenylmethane leuco bases studied inhibit phagocytosis (Table I), only 33 per cent of the total number of compounds showing a normal count in the highest dilution used. In analyzing this inhibition, consistent correlation with bactericidal potency and toxicity is hard to follow.

For instance, leucomalachite green (D 1) and leucobenzein (D 8) have the same strength as regards bactericidal action, killing *Staphylococcus aureus* at 1:400. But D 1, causing a 60 per cent reduction of the phagocytic index at 1:40,000, permits an approximately normal count at 1:160,000, while D 8 at 1:600,000 shows an 80 per cent inhibition of this activity. The maximum non-lethal dose for D 1

[8] Cross, H. B., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 350.

is 1.0 mg., for D 8, 2.5 mg., so that D 1, while two and one-half times as toxic and having the same bactericidal action, allows a much greater degree of phagocytosis than D 8.

p-Hydroxyleucomalachite green (D 2) and *p*-aminoleucomalachite green (D 3) show divergence in another direction. Both have a maximum non-lethal dose of 1.0 mg. D 3 exerts no bactericidal power at 1:400, while D 2 kills at that dilution. At 1:160,000, D 2 exhibits an approximately normal index, D 3 a 33 per cent reduction. At 1:320,000, D 2 shows a 50 per cent reduction and D 3 only a 25 per cent.

A third variation in effect is seen between *o*-hydroxyleucomalachite green (D 9) and *o*-methoxyleucomalachite green (D 11). The phagocytic indices are reduced 80 per cent by both chemicals at 1:320,000, but neither has any bactericidal power at 1:400, and their maximum non-lethal doses differ widely, D 9 being two and one-half times as toxic as D 11.

Triphenylmethane Dyes.

In comparing the specific dyes with their leuco bases the same irregularity is found. Neither *o*-hydroxymalachite green (D 7) nor its leuco base (D 9) in 1:400 possesses bactericidal power; D 9, however, is two and one-half times as toxic, and at 1:600,000 reduces the phagocytic index 90 per cent as compared with 25 per cent by D 7 in the same dilution (Table II).

In the case of the *p*-methoxymalachite green (D 12) and its leuco base (D 10), the dye is more toxic and bactericidal than the leuco base, but from 1:15,000 to 1:1,500,000, D 12 shows a normal phagocytic index, while at 1:600,000, D 10 reduces it 66 per cent. It may be noted here that *p*-methoxymalachite green (D 12) and ethylviolet chloride (D 32) are exceptional in allowing normal indices in a bactericidal dilution.

TABLE I.

Triphenylmethane Leuco Bases.

Chemical.	Maximum non-lethal dose intraperitoneally.		Bactericidal for staphylococci in whole blood in 2 hrs.	Effect on phagocytic index of different dilutions of chemicals.				
	Mg.	Dilution in mouse.		1:40,000	1:80,000	1:160,000	1:320,000	Control.
(D 1) Leucomalachite green.	1.0	1:3,000	0 at 1:400	3.5	3.8	8.6	11.0	9.9
(D 3) *p*-Aminoleucomalachite green.	1.0	1:3,000	∞ " 1:400	3.2	4.1	6.0	7.1	9.9
(D 6) *p*-Methylleucomalachite green.	2.5	1:1,200		4.4	4.0	7.3	4.4	9.9
				1:1,500	1:15,000	1:150,000	1:1,500,000	Control.
(D 13) *p*-Ethoxyleucomalachite green.	0.5	1: 6,000	∞ at 1:400	3.6	5.0	5.0	6.2	6.0
				1:40,000	1:80,000	1:160,000	1:320,000	Control.
(D 2) *p*-Hydroxyleucomalachite green.	1.0	1:3,000	+ at 1:400	3.2	5.9	8.8	6.4	9.9
				1:600	1:6,000	1:60,000	1:600,000	Control.
(D 9) *o*-Hydroxyleucomalachite green.	0.5	1:6,000	∞ at 1:400	2.4	2.7	2.6	2.5	12.3
				1:1,500	1:15,000	1:150,000	1:1,500,000	Control.
(D 14) 2,4-Dihydroxyleucomalachite green.	1.25	1:2,400	∞ at 1:400	2.8	3.9	6.6	4.3	6.4
				1:600	1:6,000	1:60,000	1:600,000	Control.
(D 11) *o*-Methoxyleucomalachite green.	1.25	1:2,400	∞ at 1:400	1.2	2.3	2.1	2.5	12.3

TABLE I—*Concluded.*

Chemical.	Maximum non-lethal dose intraperitoneally.		Bactericidal for staphylococci in whole blood in 2 hrs.	Effect on phagocytic index of different dilutions of chemicals.				
	Mg.	Dilution in mouse.		1:40,000	1:80,000	1:160,000	1:320,000	Control.
(D 4) Leucocrystal violet.	1.0	1:3,000	∞ at 1:350	2.4	1.2	4.4	3.4	9.9
				1:600	1:6,000	1:60,000	1:600,000	Control.
(D 10) *p*-Methoxyleucomalachite green.	2.5	1:1,200	0 at 1:400	4.5	3.2	2.4	4.2	12.3
(D 8) Leucobenzein.	2.5	1:1,200	0 " 1:400		3.2	2.6	2.0	12.3

The blanks in the tables indicate that the cells in those dilutions were too disintegrated to count.

In general, the leuco compounds are less bactericidal than the dyes, the difference being quite marked in some cases.

(D 18) Malachite green nitrate..... 1 : 6,400
(D 1) Leucomalachite green..... >1 : 400
(D 16) *p*-ethoxymalachite green chloride..... 1 : 12,800
(D 13) *p*-ethoxyleucomalachite green chloride..... >1 : 400
(D 15) *o*-methoxymalachite green nitrate..... 1 : 6,400
(D 11) *o*-methoxyleucomalachite green nitrate.....>1 : 400

This bactericidal relationship between the dye and its corresponding leuco base is not found in regard to the antiphagocytic action. Without exception both leuco bases and dyes decrease the phagocytic index, and the dilution represented by the largest non-lethal dose in the mouse is more leucocytotropic than bacteriotropic.

Acridines.

The acridines represented in Table III yield findings very similar to that with the triphenylmethane dyes, although as a group they are not so bactericidal, nor is perhaps the antiphagocytic action so great. In comparing the chemicals of this group, proflavine (D 26) stands out as possessing almost ideal characteristics, very similarly to *p*-methoxymalachite green (D 12) and ethyl violet (D 32) of the triphenylmethane dyes; the tropic relationships of the chemicals are such that a dose non-lethal for mice is also bactericidal for staphylo-

TABLE II.

Triphenylmethane Dyes.

Chemical.	Maximum non-lethal dose intraperitoneally.		Bactericidal for staphylococci in whole blood in 2 hrs.	Effect on phagocytic index of different dilutions of chemicals.				
	Mg.	Dilution in mouse.		1:600	1:6,000	1:60,000	1:600,000	Control.
(D 18) Malachite green nitrate.	0.6	1:5,000	0 at 1:6,400	0.24	—	11.5	15.0	14.1
				1:1,200	1:12,000	1:120,000	1:1,200,000	Control.
(D 16) *p*-Ethoxymalachite green chloride.	0.6	1:5,000	0 at 1:12,800	—	1.9	2.8	—	4.2
(D 19) *p*-Hydroxymalachite green chloride.	0.3	1:9,000	∞ " 1:1,600	—	—	1.8	2.3	4.0
				1:600	1:6,000	1:60,000	1:600,000	Control.
(D 7) *o*-Hydroxymalachite green.	1.25	1:2,400	∞ at 1:400	1.8	2.1	3.0	3.0	4.1
				1:1,500	1:15,000	1:150,000	1:1,500,000	Control.
(D 15) *o*-Methoxymalachite green nitrate.	0.6	1:5,000	0 at 1:6,400	4.1	4.4	6.8	5.0	6.4
				1:40,000	1:80,000	1:160,000	1:1,320,000	Control.
(D 5) Hexamethylviolet (crystal).	1.0	1:3,000	0 at 1:350 24 hrs. 0 at 1:39,400	0.0	3.0	8.8	4.4	7.4
				1:900	1:9,000	1:90,000	1:900,000	Control.
(D 20) *p*-Tolylmalachite green chloride.	0.03	1:90,000	0 at 1:2,000	—	—	—	—	9.1

TABLE II—*Concluded.*

Chemical.	Maximum non-lethal dose intraperitoneally.		Bactericidal for staphylococci in whole blood in 2 hrs.	Effect on phagocytic index of different dilutions of chemicals.				
	Mg.	Dilution in mouse.		1:1,200	1:12,000	1:120,000	1:1,200,000	Control.
(D 17) *o*-Chloromalachite green chloride.	0.6	1:5,000	0 at 1:1,600	—	0.8	3.4	2.3	3.4
				1:600	1:6,000	1:60,000	1:600,000	Control.
(D 24) *p*-Chloromalachite green nitrate + 3.5 H_2O.	0.5	1:6,000	+ at 1:2,500	—	1.3	2.2	3.2	7.1
(D 21) Brilliant green nitrate + 1 H_2O.	0.003	1:900,000	0 " 1:4,000	3.1	5.0	3.3	7.9	7.8
(D 32) Ethylviolet chloride	0.125	1:24,000	0 " 1:8,000	4.5	13.0	12.0	13.0	12.3
(D 31) 3, 4-Methylene-dihydroxymalachite green chloride + 4 H_2O.	0.06	1:50,000	0 " 1:8,000	6.8	11.0	7.0	12.5	12.3
(D 27 a) 2-Ethoxy-4', 4"-bisdimethylaminotriphenylcarbinol.	0.03	1:90,000	0 " 1:500	—	1.0	—	9.0	14.1
(D 27 b) *o*-Ethoxymalachite green.	1.25	1:2,400	0 " 1:500	—	—	—	—	14.1
(D 22) *p*-Nitromalachite green chloride.	0.03	1:90,000	+ " 1:2,000	—	1.3	5.5	4.8	7.1
				1:1,500	1:15,000	1:150,000	1:1,500,000	Control.
(D 12) *p*-Methoxymalachite green.	0.5	1:6,000	0 at 1:3,200	4.5	8.0	7.5	8.5	7.6

coccus and in this concentration not antiphagocytic. Since the introduction of acriflavine by Ehrlich[9] members of this group of chemicals have been employed in the treatment of various infections.

Browning and Cohen[10] have recently reported on the antiseptic properties of a rather complete series of acridines, and although not all derivatives were the same

[9] Ehrlich, P., and Benda, L., *Ber. chem. Ges.*, 1913, xlvi, 1931.

[10] Browning, C. H., and Cohen, J. B., *Brit. Med. J.*, 1921, ii, 695.

TABLE III.

Acridines.

Chemical.	Maximum non-lethal dose intraperitoneally.		Bactericidal for staphylococci in whole blood in 2 hrs.	Effect on phagocytic index of different dilutions of chemicals.				
	Mg.	Dilution in mouse.		1:600	1:6,000	1:60,000	1:600,000	Control.
(D 54) 3, 6-Dihydroxy-acridine.	1.25	1:2,400	∞ at 1:1,000	0.5	5.2	7.9	15.0	10.3
(D 53) 3, 6-Diamino-9-phenylacridine.	1.25	1:2,400	∞ " 1:1,000	—	1.3	0.5	1.6	10.3
(D 60) Diamino-*n*-methyl-acridone.	1.25	1:2,400	∞ " 1:500	2.5	4.5	11.3	7.1	15.1
(D 43) 9, 10-Dimethyl-acridinium chloride.	0.625	1:5,000	∞ " 1:500	—	3.0	6.1	6.1	12.3
				1:200	1:2,000	1:20,000	1:200,000	Control.
(D 34) 3, 6-Diamino-10-methylacridinium chloride + 1 H_2O.	0.5	1:6,000	0 at 1:1,000	0.8	3.9	7.2	6.2	12.3
				1:600	1:6,000	1:60,000	1:600,000	Control.
(D 57) 2-Amino-6-hydroxy-acridine dihydrochloride.	2.5	1:1,200	∞ at 1:500	—	3.0	1.2	1.2	10.3
(D 26) Diaminoacridine sulfate + 3 H_2O.	2.5	1:1,200	0 " 1:500	1.7	17.8	14.0	13.0	14.1
(D 49) Leucocyanotrypaflavine dihydrochloride.	0.312	1:9,000	+ " 1:500	1.8	5.6	9.2	9.6	10.3
(D 42) Cyanotrypaflavine.	0.078	1:38,400	0 " 1:500	4.0	6.4	2.6	8.7	12.3
(D 56) Acridine orange dihydrobromide.	0.625	1:5,000	∞ " 1:500	—	—	1.9	3.2	10.3
(D 40) Homoflavine ("acridine yellow").	0.6	1:5,000	∞ " 1:1,000	—	4.5	5.5	7.8	12.3

as those given here, confirmation was made in respect to bactericidal action on staphylococcus. These authors did not study the influence of the group on the phagocytic index. Gay and Morrison[11] using acriflavine claim negative chemotherapeutic results in experimental streptococcus empyema in rabbits, regardless of

[11] Gay, F. P., and Morrison, L. F., *J. Infect. Dis.*, 1921, xxviii, 1.

the high bactericidal potency of the drug against this organism. They show phagocytosis to be inhibited by strong concentrations, such as have usually been employed, but state that the dye sterilizes considerable quantities of pus in the test-tube in a dose which does not inhibit phagocytosis.

Browning, Gulbransen, Kennaway, and Thornton[12] report that acriflavine kills staphylococcus in a dilution of 1:100,000 in serum and does not inhibit phagocytosis above 1:500. It is difficult to compare our results with theirs, as a different medium was used, the incubation period was shorter in performing the bactericidal test, they did not consider phagocytosis inhibited unless the inhibi-

TABLE IV.

Safranines.

Chemical.	Maximum non-lethal dose intraperitoneally.		Bactericidal for staphylococci in whole blood in 2 hrs.	Effect on phagocytic index of different dilutions of chemicals.				
	Mg.	Dilution in mouse.		1:600	1:6,000	1:60,000	1:600,000	Control.
(D 63) Methylnaphthophenazonium chloride.	0.156	1:19,200	+ at 1:500	—	—	9.7	8.6	15.1
(D 62) Isorosindulin nitrate.	2.5	1:1,200	+ " 1:500	—	2.4	—	—	15.1
(D 37) Phenosafranine chloride.	0.078	1:38,400	+ " 1:500	1.8	8.5	7.1	9.0	12.3
				1:200	1:2,000	1:20,000	1:200,000	Control.
(D 47) Phenylrosindulin chloride.	0.625	1:4,800	0 at 1:500	0.8	1.3	2.8	2.1	12.3

tion exceeded 50 per cent, and they used human materials in their method. Despite the toxic action of proflavine and acriflavine for the phagocyte, they seem to have some local therapeutic action. According to Davis[13] both of these chemicals, following either an intravenous injection or *per os* administration, render the urine bactericidal for both staphylococcus and the colon bacillus. Davis and Harrell[14] also report a therapeutic action of acriflavine in treatment of gonorrheal urethritis.

Safranines.

The small number of safranines studied does not warrant any general deductions as to this group of compounds. But for the staphylo-

[12] Browning, C. H., Gulbransen, R., Kennaway, E. L., and Thornton, L. H. D., *Brit. Med. J.*, 1917, i, 73.

[13] Davis, E. G., and Beck, G. H., *J. Urol.*, 1921, v, 215.

[14] Davis, E. G., and Harrell, B. E., *J. Urol.*, 1918, ii, 257.

coccus, the chemicals shown in Table IV exert a very low bactericidal action. And they are markedly antiphagocytic with the exception of phenosafranine, which produces a 25 per cent reduction in the phagocytic index in a dilution of 1:600,000.

TABLE V.

Phenazines.

Chemical.	Maximum non-lethal dose intraperitoneally.		Bactericidal for staphylococci in whole blood in 2 hrs.	Effect on phagocytic index of different dilutions of chemicals.				
	Mg.	Dilution in mouse.		1:600	1:6,000	1:60,000	1:600,000	Control.
(D 65) *unsym.*-Diaminophenazine hydrochloride.	1.25	1:2,400	∞ at 1:500	—	6.3	5.6	10.6	15.5
(D 38) *unsym.*-Diaminosafranine hydrochloride.	2.5	1:1,200	∞ " 1:500	—	2.8	6.5	8.9	12.3
				1:200	1:2,000	1:20,000	1:200,000	Control.
(D 46) *sym.*-Diaminophenazine hydrochloride.	1.25	1:2,400	+ at 1:500	—	2.8	2.9	4.0	12.3
				1:600	1:6,000	1:60,000	1:600,000	Control.
(D 36) Toluylene red hydrochloride.	1.25	1:2,400	∞ at 1:500	3.2	6.0	3.3	8.4	12.3
				1:200	1:2,000	1:20,000	1:200,000	Control.
(D 44) Dimethylnaphthoenrhodine hydrochloride.	0.6	1:5,000	∞ at 1:500	—	3.5	6.5	5.0	12.3

Phenazines and Quinones.

The phenazines (Table V), and quinones (Table VI) both may be classed as drugs that are not bactericidal for staphylococci, but they are antiphagocytic in dilutions non-toxic for a mouse, with the exception of sodium chloranilate in the quinone group. This compound apparently has no bactericidal action, yet permits of phagocytosis within 25 per cent of normal in a dilution of 1:600.

TABLE VI.

Quinones.

Chemical.	Maximum non-lethal dose intraperitoneally.		Bactericidal for staphylococci in whole blood in 2 hrs.	Effect on phagocytic index of different dilutions of chemicals.				
	Mg.	Dilution in mouse.		1:600	1:6,000	1:60,000	1:600,000	Control.
(D 58) Quinone.	0.625	1:5,000	∞ at 1:1,000	4.0	2.3	1.0	4.2	10.3
(D 59) *p*-Nitrosophenol.	0.312	1:9,000	+ " 1:50	2.3	3.2	5.6	13.0	15.5
(D 64) Sodium chloranilate.	5.0	1:600	∞ " 1:500	11.1	14.2	14.2	2.0	15.1
(D 67) Anilino-β-naphtha quinone.	1.25	1:2,400	∞ " 1:1,000	—	6.0	12.0	7.7	10.3

Cinchonas.

The cinchona compounds in Table VII are the members of a group of alkaloids on which we reported in a previous paper.[1] It was found that the aromatic compounds had the power of killing, rapidly, multiple lethal doses of virulent pneumococci when organisms and drugs were injected simultaneously into the peritoneal cavity of mice. In this respect, all cinchona derivatives were found to be superior to optochin, but the different aromatic substitution products were possessed of varying degrees of bactericidal action *in vitro* and *in vivo*. Intravenous treatment with these drugs lowered the resistance of the mice as did any method of treatment except *per os*, when given at a site other than the one at which the organisms had been injected. Although mice with definitely established pneumococcus infection were not cured, these compounds, under specified conditions, had a measurable amount of protective action, greater perhaps than that of any drug used heretofore against a bacterial infection.

At a glance the compounds in therapeutic doses in Table VII are seen to possess marked antiphagocytic activity, and with the possible exception of C 29, the aromatic compounds are seen to be more leucocytotropic than optochin. The question naturally arises, whether this antiphagocytic property is the cause of the therapeutic failure of the chemicals in an established infection.

TABLE VII.

Cinchonas.

Chemical.	Maximum non-lethal dose intraperitoneally.		Bactericidal for staphylococci in whole blood in 2 hrs.	Effect on phagocytic index of different dilutions of chemicals.			
	Mg.	Dilution in mouse.		1:1,000	1:2,000	1:4,000	Control.
(C 29) Hydroquinine chloroacetyl-anilide.	0.5	1:6,000		—	6.0	3.7	7.7
(C 36) Dihydroquinine *p*-chloro-acetylaminophenol hydrochloride.	2.5	1:1,200	+ at 1:1,000	—	—	2.4	7.7
(C 40) Dihydroquinine *m*-chloro-acetylaminophenol hydrochloride.	1.5	1:1,900	+ " 1:2,000	1.9	3.2	∞	7.7
(C 110) Dihydroquinine 4-chloro-acetylaminopyrocatechol hydrochloride.	4.0	1:750	+ " 1:500	—	1.2	0.72	7.7
(C 11) Optochin (ethylhydrocupreine).	3.0	1:960	+ " 1:500	5.2	1.8	3.36	7.7
(C 9) Hydroquinine.	1.0	1:3,000		4.6	6.0	4.0	7.7

Bordet's experiments[15] showing that the resistance of an animal is lowered after it has been partially depleted of leucocytes by means of carmine, and also Lippman's work[16] proving that optochin has no protective action in animals whose leucocyte count has been lowered by thorium, may be cited in support of the postulate that a drug which inhibits the action of the leucocytes will have little value in treatment of a bacterial disease. However, the experiments of neither Bordet[15] nor Lippman[16] are conclusive for the reason that the chemicals used to decrease the number of white cells may have exerted a toxic action on other functions vital to the resistance of the animal. Acton[17] has demonstrated that a number of cinchona derivatives inhibit completely the migration of leucocytes in a dilution of 1:500. The classical work of Binz[18] shows that quinine itself, along with its other general protoplasmic toxicities, inhibits phagocytosis.

[15] Bordet, J., Studies in immunity, translated by Gay, F. P., New York, 1909, 30.

[16] Lippman, *Z. Immunitätsforsch., Orig.*, 1915–16, xxiv, 107.

[17] Acton, H. W., *Lancet*, 1922, i, 124.

[18] Binz, C., Das Chinin. Nach den neuern pharmakologischen Arbeiten dargestellt, Berlin, 1875.

With the four cinchona derivatives, as with optochin, we have been able to show that migration of leucocytes follows almost immediately after the injection of a non-toxic dose of the drug into the peritoneal cavity of a mouse. The experiments reported in Table VIII were carried out by injecting 0.5 mg. of drug in a 1 cc. volume into the peritoneum of mice. At 1, 2, 3, 4, and 24 hours respectively, a small quantity of fluid was removed, diluted with a known amount of Turk's solution, shaken, and counted in the usual manner. Obviously, the drugs have a positive chemotactic influence on leucocytes in mice, since these migrate to the site of injection. Inasmuch as the cinchona compounds, as has been shown above, inhibit the phagocy-

TABLE VIII.

Chemotactic Influence of Aromatic Cinchona Derivatives.

Chemical.	Duration of observation.				
	1 hr.	2 hrs.	3 hrs.	4 hrs.	24 hrs.
(C 29) Hydroquinine chloroacetylanilide.	2,110*	2,340	5,740	4,000	9,400
(C 36) Dihydroquinine *p*-chloroacetylamino-phenol hydrochloride.	3,520	5,360	5,140	4,300	No fluid.
(C 40) Dihydroquinine *m*-chloroacetylamino-phenol hydrochloride.	2,720	3,020	4,620	5,000	" "
(C 110) Dihydroquinine 4-chloroacetylamino-pyrocatechol hydrochloride.	3,860	4,200	6,800	9,840	63,680
(C 11) Optochin (ethylhydrocupreine).	740	1,660	22,300	5,360	No fluid.

* The numbers refer to cells per c.mm. of fluid.

tosis of staphylococcus and are also proven to have positive chemotactic characteristics, it would seem that the antiphagocytic action is due, to a large extent, to the paralysis of the function of the leucocytes and not to destruction of the cells.

DISCUSSION AND SUMMARY.

We are dealing, as the results show, with groups of chemicals, all of which, whether bacteriotropic or not, greatly inhibit the engulfing of *Staphylococcus aureus* by leucocytes. Not a sufficiently large number of experiments was performed in attempt to cure experimental staphylococcus infections to warrant any conclusion in regard to possible therapeutic activity against this organism. How-

ever, as will appear in another paper, the only group out of the seven which definitely possessed an *in vivo* bactericidal action against pneumococcus is that of the cinchona derivatives. Certain members of the other chemical groups studied, although bactericidal in a very high dilution,—chemicals in which the concentration of a non-lethal dose was many times greater than that required to kill multiple minimal lethal doses of organisms *in vitro*,—had no certain effect when bacteria and drug were injected simultaneously into the peritoneal cavity of a mouse. In fact, the treated mouse often died before the controls.

If we may assume,—leaving out of consideration the practical significance of *in vivo* chemical destruction and excretion following the injection of the drug into the animal,—that the failure of these chemicals to exhibit a benign influence on a systemic infection in cases in which the drug can be used in a bactericidal dilution, is due to their antiphagocytic property, only one step has been taken in analysis of the factors vital for the defense of the animal against a specific microorganism. Why do these chemicals inhibit leucocytic activity? Is it because of their influence upon complement, opsonin, or the leucocyte itself, or some special one function that determines the ability to ingest bacteria? Only further work can definitely settle this question and also determine whether or not such an analysis would be of practical importance in a rational development of chemotherapy.

The ideal chemotherapeutic agent may be one that has an *in vivo* bactericidal potency and a negligible or stimulatory phagocytic action in doses non-lethal for the experimental animal. However difficult such a drug may be to find, it seems unlikely that the ultimate success in chemotherapy will be so simple. Again, it is conceivable that a secondary action of a drug, although leucocytotropic and not bacteriotropic, may bring about conditions in the animal body that will enable it to throw off the invading organism. Or finally, a drug compatible with the forces necessary to the host's defense and possessing *in vivo* bactericidal action to a greater or less degree may be the chemical sought for, the goal toward which we should strive, to achieve a rational chemotherapy for infectious diseases.

CONCLUSIONS.

With certain members of the triphenylmethane dyes and leuco bases, safranines, phenazines, quinones, and cinchona groups of chemicals, there exists no consistent parallelism between the bacteriotropic activities and the organotropic and leucocytotropic activities.

All the chemicals tested possess a leucocytotropic action, as measured by the decreased ability of leucocytes to ingest staphylococci. This action against the functional activity of the leucocyte is more pronounced than the organotropism or bacteriotropism (for staphylococcus).

Four aromatic cinchona compounds, hydroquinine chloroacetylanilide hydrochloride (C 29), dihydroquinine *p*-chloroacetylaminophenol hydrochloride (C 36), dihydroquinine *m*-chloroacetylaminophenol hydrochloride (C 40), dihydroquinine 4-chloroacetylaminopyrocatechol hydrochloride (C 110), and optochin (C 11) are markedly antiphagocytic in their therapeutic dose. They possess a positive chemotactic action for leucocytes when injected into the peritoneal cavity of mice.

In the cases of *p*-methoxymalachite green (D 12), ethyl violet chloride (D 32), and diaminoacridine sulfate (D 26) the condition was approached in which the concentration of a non-lethal dose for mice is staphylotropic and not leucocytotropic.

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RABBIT SEPTICEMIA BACILLUS, TYPES D AND G, IN NORMAL RABBITS.

BY PAUL H. DE KRUIF, PH.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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INTRODUCTION.

In preparation for experiments on the epidemiology of rabbit septicemia, it was necessary to obtain rabbits which were free from infection with this organism. The normal rabbit stock of the laboratory consequently was examined by culturing of nasal mucous membranes. During the routine of this examination the following interesting observations were made.

EXPERIMENTAL.

Procedure for Detection of Type D.—A small sterile swab was carefully introduced into the nostril of the rabbit and subsequently streaked at once on 10 per cent horse serum agar plates, pH 7.4. The plates were incubated at 37°C. for 24 hours and examined.

Of the first series of twenty-nine animals examined, fifteen were found to harbor typical D organisms. These animals were healthy to all outward appearance. They were well nourished, of good appetite, and had no visible discharge from the nose. The Type D colonies were present in great abundance. In many of the rabbits they constituted 50 per cent or more of the nasal flora. The Type D are easily differentiated from other colonies by their high fluorescence in artificial light, their rather small size, smoothness of contour, and by the fact that they are very "soft" and tend to disintegrate completely when gently touched with a platinum wire. Other fluorescent colonies can either be shoved about on the agar surface, retaining their form perfectly, or break up into two or three distinct fragments.

Suspected Type D colonies are examined by hanging drop, oil immersion objective, and if found to yield non-motile, minute coccoid

rods, are subcultured to rabbit serum broth. These cultures are then examined for turbidity, bipolarity in Löffler's methylene blue stain, acid agglutination optimum, and agglutination by specific anti-D serum.

Type G in Normal Rabbits.

In the same series of twenty-nine animals, four nasal swabs yielded plates heavily seeded with colonies exactly resembling those of the mutant G form.[1] These colonies were translucent and of faintly bluish tinge, exhibited no fluorescence with artificial light, and had slightly irregular borders.

They were fished into serum broth and yielded the granular sedimenting growth characteristic of Microbe G, rabbit septicemia bacillus. The organisms were minute, non-motile, coccoid bacilli, growing singly or in pairs. In hanging drop, fresh preparation, they exhibited the typical shadows at the poles. Löffler's methylene blue demonstrated their bipolarity.

TABLE I.

Acid Agglutination Optimum of Suspected Type G.

pH.....	6.6	6.3	6.0	5.5	5.3	5.0	4.6	3.9	3.3	3.0
Result.	Tr.	+	++	++	++	++	C.	C.	+	Tr.

These results justified more minute examination, since it would be important to find out whether Type G, up till now observed only as a mutant from Type D, under cultural conditions, occurred naturally in the animal body.

Experiment 1. Acid Agglutination Optimum of Suspected Type G.—Organisms from a suspected Type G colony were planted in serum broth, and the 16 hour granular culture that resulted was washed four times and finally suspended in distilled water. 1 cc. quantities of this suspension were then mixed with 1 cc. of buffer solution of different hydrogen ion concentrations. The buffer solution used was glycocoll-Na acetate-Na_2HPO_4, $\frac{M}{1/2}$ concentration. Incubation 43°C. for 14 hours. The result is recorded in Table I.

The broad zone of hydrogen ion concentration over which agglutination took place is characteristic of Type G recovered from the

[1] De Kruif, P. H., *J. Exp. Med.*, 1922, xxxv, 561.

animal body in artificial infections.[2] The agglutination reaction by specific serum was next attempted.

Experiment 2. Agglutination of Suspected Type G by Rabbit Serum > D.—It has been observed in a preceding paper[3] that Types D and G show antigenic community, and that inoculation with Type D results in a slightly stronger agglutinin response than that following injection of Type G. Rabbit serum anti-D was therefore used in this experiment. Observation of Table I shows that marked agglutination of the suspected organism occurs at pH 6.0. This fact makes it impossible to perform the agglutination reaction with 0.85 per cent NaCl dilutions of serum, since these would cause agglutination of the organism in dilutions no longer protected by the buffer action of the serum. It is apparent that, in a series of dilutions of serum with 0.85 per cent NaCl, the pH would vary from 7.4 in the concentrated, to 5.8–6.0 in the high dilutions.

TABLE II.

Agglutination of Suspected Type G by Anti-D Immune Serum.

Serum.	Agglutination.									
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Control. No serum.
Rabbit serum, anti-D.....	C.	C.	C.	++	−	−	−	−	−	−
Normal rabbit serum.....	++	+	+	−	−	−	−	−	−	−

The immune serum for this test was therefore diluted in glycocoll-Na acetate, Na_2HPO_4 buffer, pH 7.1. This insured the occurrence of a hydrogen ion concentration at which suspensions of the suspected organism were stable. The results of the agglutination test are given in Table II. Incubation, as usual, at 43°C. for 14 hours.

This experiment and other similar ones definitely identify the suspected organism as a member of the rabbit septicemia group. It remained to test its virulence on intrapleural injection into rabbits.

Experiment 3. Lack of Virulence of Suspected Type G.—A 6 hour serum broth culture of the suspected organism was carefully shaken up to secure uniform distribution of clumps, diluted to 5×10^{-2} cc. in broth. Amounts equivalent to 0.05 and 0.1 cc. of the original culture were inoculated intrapleurally into young rabbits of 600 gm. weight. No untoward result was discovered, although the animals were kept under observation for more than 30 days. This lack of virulence confirms the suspicion of the identity of this organism with the mutant G forms.

[2] De Kruif, P. H., *J. Gen. Physiol.*, 1922, iv, 387.

[3] De Kruif, P. H., *J. Exp. Med.*, 1921, xxxiii, 773.

Subsequent examination of supposedly normal adult rabbits has revealed a fair proportion as carriers of this organism, which conforms in every characteristic to the G type arising from Type D by mutation in culture.

Experiment 4. Virulence of Type D Isolated from Nasal Mucous Membranes of Normal Rabbits.—It has been remarked in a preceding paragraph that fifteen out of twenty-nine rabbits of the normal stock were found to harbor typical Microbe D. It was considered of interest to test the virulence of these organisms, since that of strains arising from fatal bronchopneumonias is invariably high.[1,3]

A Type D culture obtained from Rabbit 1 was accordingly seeded into 10 per cent rabbit serum broth and the virulence of the resulting 6 hour culture tested by intrapleural injection into young rabbits of 600 gm. weight. The technique of the test was identical with that described in a preceding paper.[3] The result is recorded in Table III.

TABLE III.

Virulence of Type D Isolated from Mucous Membranes of Apparently Healthy Rabbits.

Rabbit No.	Weight of rabbit.	Age of culture.	Amount injected intrapleurally.	Result.	Necropsy findings.
	gm.	*hrs.*	*cc.*		
2	605	6	1×10^{-4}	Died in 36 hrs.	Typical fibrinopurulent pleuritis, pericarditis, and bronchopneumonia.
3	610	6	1×10^{-5}	" " 48 "	" "
4	610	6	1×10^{-4}	" " 54 "	" "
5	600	6	1×10^{-3}	" " 36 "	" "

This experiment was made on November 22, 1921. At the present date, March 31, 1922, Rabbit 1 from which this highly virulent Type D culture was isolated, is alive, of full weight, and good appetite, and still harbors Type D organisms. Similar results have been obtained with Type D isolated from other normal rabbits.

This observation is of fundamental importance in the epidemiological study of the bronchopneumonia of rabbits caused by the rabbit septicemia bacillus. It is clear that these animals may harbor on the nasal mucous membrane organisms which display a virulence, by intrapleural test, equal to that of strains recovered from fatal infections. It would appear, from this and other considerations to be revealed in later communications, that there is no strict relation-

ship between intrapleural virulence and invasibility occurring in natural infections. In a word, some resistance-lowering factor must be present to enable the virulent organism to produce the fatal bronchopneumonia.

Agglutinins in the Blood of Rabbits Naturally Infected with Types D and G.

Since it is possible for rabbits to harbor highly virulent Type D organisms on the nasal mucous membrane, and remain for a long time without signs of disease, it was considered important to discover whether the blood showed any evidence of immune substances against the rabbit septicemia bacillus.

TABLE IV.

Relation between Agglutination Titer and Presence of Types D and G on the Nasal Mucous Membrane.

14 hours at 43°C.; pH 7.1.

Rabbit No.	Examination of nasal mucous membrane.	Agglutination. Dilution of serum + Type G at pH 7.1. 1:10*	1:20	1:40	1:80	1:160	1:320	Control.	Titer of serum.†
6	No Type D or G present.	+	Tr.	Tr.	—	—	—	—	< 1:10
7	" " " " " "	+	—	—	—	—	—	—	< 1:10
8	" " " " " "	++	+	Tr.	Tr.	—	—	—	< 1:10
9	" " " " " "	++	+	—	—	—	—	—	< 1:10
10	" " " " " "	+	Tr.	Tr.	Tr.	Tr.	—	—	< 1:10
11	" " " " " "	+	"	"	—	—	—	—	< 1:10
12	" " " " " "	++	++	++	+	+	Tr.	—	< 1:10
13	Type D present.	C.	C.	++	Tr.	—	—	—	1:20
4	" G "	"	"	C.	C.	+	—	—	1:80
14	No Type D or G present.	Tr.	Tr.	—	—	—	—	—	< 1:10
15	Type D present.	C.	C.	C.	++	+	—	—	1:40
16	No Type D or G present.	Tr.	—	—	—	—	—	—	< 1:10
17	" " " " " "	+	Tr.	—	—	—	—	—	< 1:10
18	Type D present.	++	++	++	++	Tr.	—	—	< 1:10

* Represents dilution of serum only. Multiply by 2 to obtain the final dilution of serum + suspension.

† The titer of the serum is read as the smallest amount causing complete agglutination of Microbe G.

Experiment 5. Relation between Agglutination Titer and Presence of Types D and G on the Nasal Mucous Membrane.—The method chosen was that described in Experiment 2. Four times washed Type G, suspended in distilled water, was tested against the suspected serum, diluted with buffer (glycocoll-Na acetate-Na_2HPO_4), pH 7.1. This method was chosen because, first, Type G is far more sensitive to rabbit septicemia agglutinins than is Type D; second, because at this pH, Type G shows itself to be perfectly stable and amenable therefore to agglutination test.

TABLE V.

Relation between Agglutination Titer and Presence of Types D and G on the Nasal Mucous Membrane.

Rabbit No.	Examination of mucous membrane.	Titer of serum.
19	No Type D or G present.	< 1:10
20	" " " " " "	< 1:10
21	" " " " " "	< 1:10
22	" " " " " "	< 1:10
23	" " " " " "	< 1:10
24	" " " " " "	< 1:10
25	" " " " " "	< 1:10
26	Type D present.	1:80
27	" " "	1:40
28	No Type D or G present.	< 1:10
29	Type G present.	1:80
30	" D "	1:40
2	No Type D or G present.	< 1:10
31	" " " " " "	< 1:10
32	" " " " " "	< 1:10
33	" " " " " "	< 1:10
34	" " " " " "	< 1:10
35	" " " " " "	< 1:10
36	Type D present.*	< 1:10

* Type D had been present for only 4 days. The animal was demonstrated to be D- and G-free for at least 2 months previous to the infection, which occurred by direct contact.

The blood was drawn from the hearts of the animals under test. The serum was diluted 1:10 in the above named buffer and then by 2's to 1:320. 1 cc. of each dilution was mixed with 1 cc. of Type G suspension—incubation at 43°C. for 14 hours. The results are summarized in Tables IV and V.

Further experiments of a similar nature are summarized in Table V. In all cases the finding "Type D or G not present" was entered

after three successive streakings on serum agar plates of mucous membrane swabs, made at 5 day intervals.

The experiments summarized in Tables IV and V show that the presence of Type D or G, bacillus of rabbit septicemia, on the nasal mucous membranes of rabbits is accompanied by distinct evidence of specific agglutinins in the blood.

Excepting in one case (Rabbit 1) complete agglutination of Type G always occurred in 1:20 or higher dilutions when Type D or G could be demonstrated on the nasal mucous membrane. On the other hand, *no* serum caused complete agglutination in 1:10 or higher when three successive streakings on serum agar plates had demonstrated the absence of Types D and G.

DISCUSSION.

These findings are important in that they demonstrate (1) that the carrier condition is associated with evidence of immunity, as determined by the presence of definite amounts of agglutinins in the blood; (2) that the serum of any rabbit, taken at random from a normal stock, is *not* to be considered normal so far as the rabbit septicemia bacillus is concerned.

SUMMARY.

Microbe G, rabbit septicemia bacillus, hitherto found only as a mutant in cultures of the rabbit septicemia bacillus, Type D, has been demonstrated to exist on the nasal mucous membranes of normal rabbits. This organism corresponds in lack of virulence, character of growth, acid agglutination optimum, and immune agglutination reaction, to the mutant G form described in previous papers.

Microbe D has been found to be present on the nasal mucous membranes of normal rabbits. These animals have survived for months with no evidence of infection other than the presence of the organism. These Type D organisms, despite their failure to cause fatal damage in their own host, are shown to possess the typical high virulence characteristic of this type, when injected intrapleurally into young rabbits.

Rabbits which are carriers of Type D or G possess a definite amount of immune agglutinins, as evidenced by test of their serum against Microbe G at pH 7.1.

Rabbits free from infection with these organisms invariably have yielded a serum which fails to agglutinate Type G completely in 1:10 or higher dilutions.

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ETIOLOGY OF YELLOW FEVER.

XIV. DURATION OF THE PROTECTIVE EFFECT OF ANTI-ICTEROIDES IMMUNE SERUM AFTER SUBCUTANEOUS INOCULATION INTO ANIMALS.

BY HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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As has already been reported,[1] the anti-*icteroides* immune serum is capable of protecting susceptible animals against infection with *Leptospira icteroides* when administered simultaneously or during the early period of the disease. Lyster, Pareja, Bailey, Vaughn, Vasconcelos, Casasus, Iglesias, Loyo, Le Blanc, Lynn and his associates, Hernandez, Lara, Villamil, Kligler, and Gann[2] have found that the mortality among human cases of yellow fever treated with the immune serum on or before the 3rd day of disease is much lower than that among untreated cases. Of 71 cases treated within the first 3 days of disease in Guatemala, Salvador, Honduras, Vera Cruz Merida, and northern Peru only 5 died (7 per cent mortality); the results of serum treatment were less favorable in the Tuxpam (Mexico) epidemic, where the mortality among the treated was 25 per cent (9 deaths among 36 cases), but here the mortality among untreated cases was correspondingly higher, being 68.6 per cent (59 deaths

[1] Noguchi, H., Serum treatment of animals infected with *Leptospira icteroides* *J. Exp. Med.*, 1920, xxxi, 159; Chemotherapy *versus* serotherapy in experimental infection with *Leptospira icteroides*, 1920. xxxii, 381. Noguchi, H., and Kligler, I. J., Immunological studies with a strain of leptospira isolated from a case of yellow fever in Merida, Yucatan, *J. Exp. Med.*, 1920, xxxii, 627; Immunology of the Peruvian strains of *Leptospira icteroides*, 1921, xxxiii, 253.

[2] Noguchi, H., Prophylaxis and serum therapy of yellow fever, *J. Am. Med. Assn.*, 1921, lxxvii, 181. See also the report of The Rockefeller Foundation for the year 1921.

among 86 cases). Of the total 187 cases treated to date, 107 received the serum on or before the 3rd day, and of these, only 14 died (mortality 13 per cent); on the other hand, there have been 41 deaths (51 per cent mortality) among 80 cases treated on or after the 4th day, and 225 deaths (56.6 per cent mortality) among 397 untreated cases occurring in the same localities during the same epidemic periods.

The serum has apparently undoubted therapeutic value in human cases of yellow fever as well as in cases of experimental infection with *Leptospira icteroides* in animals. Vaccination by means of killed cultures of *Leptospira icteroides* (injected in two subcutaneous injections of 2 cc. each, 4 to 6 days apart) has been shown to confer complete protection within 10 to 15 days of the last inoculation.[2,3] During the period required for development of active immunity, however, anti-*icteroides* serum might be utilized for the immediate protection of non-immune individuals who find themselves in an epidemic or endemic focus of yellow fever, or it might be substituted for vaccination in the case of persons who intend only to pass through an infected district. It is well known that an immune serum or antitoxin, when introduced into the system of a non-immune individual, will protect against infection for a period which varies from a few to many days according to the number of units of immune bodies initially introduced and the rate of elimination of such bodies from the inoculated individual. The passive immunity thus conferred is necessarily of short duration, yet the employment of anti-*icteroides* serum for the temporary protection of non-immune individuals might be of considerable practical value.

In the experiments to be reported here guinea pigs were used to determine the duration of the protective effect of an injection of anti-*icteroides* immune serum.

EXPERIMENTAL.

Six different doses of immune serum (0.00001, 0.0001, 0.001, 0.01, 0.1, and 1 cc.) were subcutaneously inoculated into six sets (two each) of guinea pigs of about 500 gm. body weight. The tests for the per-

[3] Noguchi, H., and Pareja, W., Prophylactic inoculation against yellow fever, *J. Am. Med. Assn.*, 1921, lxxvi, 96.

sistence of passive immunity were made 1, 2, 3, 4, 5, 7, 10, and 15 days after the injection of the immune serum. In order to infect all the guinea pigs, including six normal control animals, with the same material at the same time, the injections of the immune serum were begun 15 days before the time selected for the test inoculation of a virulent strain of *Leptospira icteroides*. The infective material used was an emulsion of the liver and kidneys of a guinea pig fatally infected with a strain of *Leptospira icteroides* isolated in Morropon by Noguchi and Kligler,[4] which killed the control guinea pigs within 9 to 10 days in quantities of 0.001, 0.01, and 0.1 cc. 0.1 cc. of the emulsion, representing about 100 minimum lethal doses, was used in this experiment and was given subcutaneously. The results are recorded in Table I.

Analysis of the table shows that the minimum quantity of anti-*icteroides* serum required for protection, as revealed by the results obtained when the serum was given an hour previous to the inoculation of the infecting material, lay between 0.00001 and 0.0001 cc. Judged from the survival of three guinea pigs which received serum 24, 48, and 72 hours previously the protective titer of the serum must be close to 0.0001 cc. There was no diminution in the protective effect within 48 hours, but after 3, 4, and 5 days 0.0001 cc. no longer gave complete protection, while 0.001 cc. protected in every instance. 7 days after the time of injection of the serum 0.001 cc. failed to protect, but 0.01 cc. was still effective. After the lapse of 10 days 0.1 cc. was required to prevent infection, and after 15 days only those animals which had received 1 cc. of the immune serum withstood infection. The rate of elimination of the immune substance in the body of the guinea pig after the subcutaneous introduction of the anti-*icteroides* serum does not proceed uniformly in the successive days following injection, but follows a characteristic course which may be roughly estimated in the manner shown in Text-fig. 1, the unit being 0.0001 cc., which neutralized at least 100 minimum lethal doses in the present series.

The rate of disappearance of the immune substance is very slow at first, but becomes rapid after about 10 days. This phenomenon is

[4] Noguchi, H., and Kligler, I. J., Experimental studies on yellow fever in northern Peru, *J. Exp. Med.*, 1921, xxxiii, 239

TABLE I.

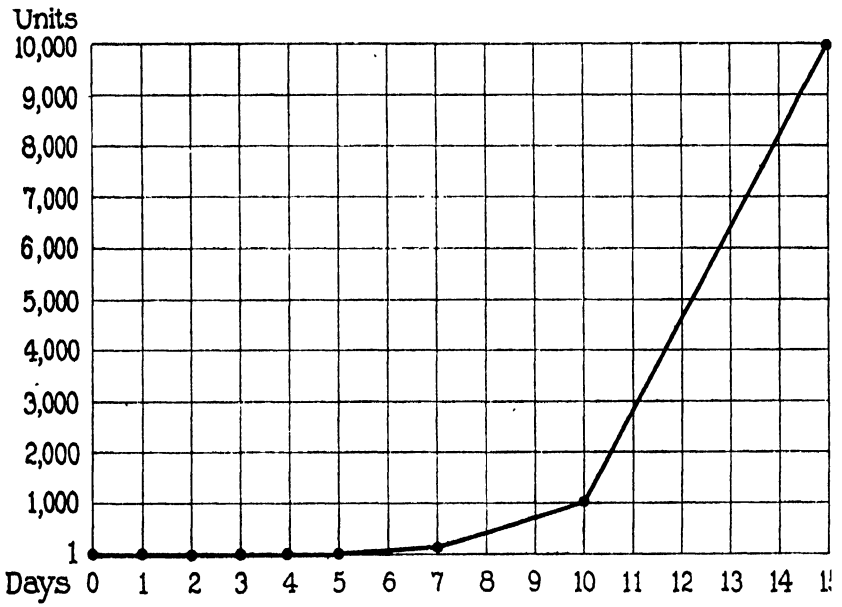
Results of Inoculation of 100 Minimum Lethal Doses of Leptospira icteroides into Guinea Pigs Previously Injected with Anti-icteroides Immune Serum.

Amount of serum injected.	Intervals between the injection of the immune serum and the inoculation of *Leptospira icteroides*.								
	1 hr.	24 hrs.	48 hrs.	3 days.	4 days.	5 days.	7 days.	10 days.	15 days.
cc.									
0.00001	+ (Killed for virus.)	−	−	−	Died in 9 days.	Died in 10 days.	Died in 11 days.	Died in 11 days.	Died in 10 days.
	+ (Recovered.)	+ (Recovered.)	Died in 9 days.	Died in 9 days.	Died in 10 days.	Died in 10 days.	Died in 13 days.	Died in 10 days.	Died in 10 days.
0 0001	−	−	−	−	+ (Killed.)	Died in 9 days.	Died in 11 days.	Died in 10 days.	Died in 10 days.
	−	−	−	+ (Recovered.)	+ (Recovered.)	Died in 11 days.	+ (Recovered.)	Died in 13 days.	Died in 11 days.
0.001	−	−	−	−	−	−	Died in 14 days.	Died in 10 days.	Died in 11 days.
	−	−	−	−	−	−	+ (Recovered.)	Died in 16 days.	Died in 12 days.
0.01	−	−	−	−	−	−	−	+ (Recovered.)	−
	−	−	−	−	−	−	−	Died in 12 days.	Died in 11 days.

0.1	−	−	−	−	−	−	−	−	Died in 11 days.
	−	−	−	−	−	−	−	−	Died in 10 days.
1.0	−	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	−	−	−

− indicates that no infection developed; + indicates the development of infection.

not characteristic of anti-*icteroides* serum alone, because various immune serums appear to undergo a similar reduction in strength when introduced into different species of animals. Knorr, for example, found that tetanus antitoxin (horse serum), given to a foreign species (guinea pigs or rabbits), is reduced rapidly and that only about $\frac{1}{100}$



TEXT-FIG. 1. Curve showing the increase in amount of anti-*icteroides* serum (in units) necessary for protection according to the length of time elapsing after administration.

of the quantity originally present in the blood can be demonstrated after 12 to 14 days.[5] Tizzoni[6] obtained similar results. Nocard[7] estimated that the longest period that the tetanus antitoxin remains

[5] Knorr, A., Das Tetanusgift und seine Beziehungen zum thierischen Organismus. Eine experimentelle Studie über Krankheit und Heilung, *Münch. med. Woch.*, 1898, xlv, 362; Die Entstehung des Tetanusantitoxins im Thierkörper und seine Beziehung zum Tetanusgift, *Fortschr. Med.*, 1897, xv, 657.

[6] Tizzoni, G., Sull' efficacia dell' antitossina nel trattamento preventivo contro il tetano dopo avvenuto l'infezione, *Gazz. osp.*, 1897, xviii, 1215.

[7] Nocard, E., Sur la sérothérapie du tétanos; essais de traitement preventif, *Bull. Acad. med.*, 1895, xxxiv, 407.

in the body is about 4 to 6 weeks. Pfeiffer and Friedberger[8] found that when an anticholera serum derived from the goat is injected into the rabbit it gives rise to an anti-immune substance by which it is neutralized in a comparatively short time. Ransom and Kitashima[9] showed that homologous tetanus antitoxin, when injected into a normal horse, may remain as long as 80 days; that is, the duration of the passive immunity is almost as long as that of active immunity. On the other hand, Jörgensen and Madsen found that not all homologous immune serums remain for a long period in the body of the injected animal, but that the length of time varies with different animal species.[10] Kraus and his coworkers failed to demonstrate any evidence for the formation of an anti-immune substance in the blood or organs of animals which had received an injection of a heterologous diphtheria antitoxin.[11] Perhaps the sudden disappearance of the immune substance after about 10 days in the present series of experiments may be intimately connected with the precipitin formation for the heterologous anti-*icteroides* horse serum. Moreover, the titer of the immune serum is certain to suffer reduction when kept at a temperature of 39°C., even *in vitro*, and may be expected to undergo similar reduction in the blood of a foreign species.

SUMMARY.

Analysis of the records of instances in which non-immune persons contracted yellow fever notwithstanding vaccination shows that the onset of disease occurs soon after vaccination, the longest period being 13 days. Since the average incubation period in yellow fever is 6 days, it seems that infection must have taken place in some instances during the period while protection was developing. These instances

[8] Pfeiffer, R., and Friedberger, E., Ueber den Verbleib der bacteriolytischen Immunkörper im tierischen Organismus nach der passiven Immunisierung, *Centr. Bakt., Ite Abt., Orig.*, 1904, xxxvii, 131.

[9] Ransom, F., The conditions which influence the duration of passive immunity, *J. Path. and Bact.*, 1900, vi, 180.

[10] Jörgensen, A., and Madsen, T., The fate of typhoid and cholera agglutinins during active and passive immunisation, Festskrift ved Indvielsen af Statens Serum-Institut, 1902, Copenhagen, Paper 6.

[11] Kraus, R., and Joachim, J., Zur Frage der passiven Immunisierung, *Wien. klin. Woch.*, 1903, xvi, 1389.

led to a study of the possibility of immediate protection by means of the anti-*icteroides* serum. It had already been shown that the immune serum protects at once against experimental *Leptospira icteroides* infection, but it remained to determine how long the protection would last.

Guinea pigs were given different quantities of the immune serum and subsequently injected, at various intervals, with a virulent strain of *Leptospira icteroides*. Complete protection enduring 5 days was obtained with as minute a quantity of serum as 0.002 cc. per 1,000 gm. of body weight. After 5 days, however, the immune substance rapidly diminished, and to keep the animal protected for as long as 10 days it was necessary to give 100 times as much, or 0.2 cc. For a man weighing 80 kilos, 0.16 cc. (0.002×80) would theoretically be sufficient to protect for at least 5 days, 1.6 cc. for 7 days, and 16 cc. for 10 days. This temporary protection may be a valuable antecedent to that furnished by vaccination, since the final effect of the latter cannot be expected until at least 9 to 10 days have passed.

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AN OUTBREAK OF MOUSE TYPHOID AND ITS ATTEMPTED CONTROL BY VACCINATION.

BY CLARA J. LYNCH, PH.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 28, 1922.)

In this paper will be described briefly an epidemic of so called mouse typhoid which prevailed with fluctuations for approximately 2½ years in a mouse breeding station maintained at The Rockefeller Institute.

The original stock of about 3,000 mice of all ages was procured by purchase and transported *en masse* to The Rockefeller Institute. The stock was especially valuable because it yielded a relatively high percentage of mice developing so called spontaneous cancers, located in the mammary glands chiefly, but also in other organs. It is known that the original stock, from which that purchased by The Rockefeller Institute was derived, had previously suffered losses from mouse typhoid. Moreover, to the stock as purchased, there were added from time to time from the outside a mouse showing a spontaneous tumor or other small accessions of healthy stock.

The transfer of the mouse stock took place in April, 1918. Nothing especially noteworthy in the fatalities arose to arrest attention until about the middle of September of that year, when an unusual number of deaths among the stock occurred.[1] From this time on, until what may be called the termination of the epidemic period, some 2½ years later, the number of deaths may be regarded as having been abnormally high, although in intervals between the wave-crests, the death rate returned to a level as low as that usually regarded as normal.

It was customary to record all deaths occurring in the stock mice and at the beginning of each month to take a census of the population

[1] The summer death rate immediately preceding the September rise was slightly higher than usual. But as during the warm summer period previously a rise had been observed, this increase was not considered significant.

which fluctuated in number roughly between 2,500 and 4,000 individuals. The numbers as given by the census varied according to the matings and to the demands made on the stock for purposes of investigation.

It was the custom also to make a gross postmortem examination of every mouse found dead. When the deaths became so frequent as to excite apprehension of an impending epidemic, bacteriological studies were made. Cultures of the spleen and liver yielded a bacillus which was identified as belonging to the group of mouse typhoid bacilli. During the course of the bacteriological studies about a dozen strains conforming to the cultural characteristics of the group were isolated. Of this number two strains were retained in culture and transplanted regularly and thus kept alive. But it was not until about a year after the epidemic began that the two strains were subjected to immunological study by Dr. Amoss.

The two strains proved to be identical in cultural and immunological reactions, and there was no reason to suppose that the first epidemic was induced by more than this one strain of a bacillus belonging to the enteritidis group. As this strain was utilized by Dr. Amoss in certain of his experiments on the production of artificial epidemics among mice,[2] its more precise biological description will be found in his papers.[3] For sake of convenience the strain has been designated Mouse Typhoid I.

Before proceeding to the more detailed account of the epidemic among the stock of cancer mice, as they were called, a brief statement regarding the conditions under which mice are propagated at The Rockefeller Institute may be of interest.

Two distinct breeding rooms or stations for mice are maintained, with separate caretakers who do not mingle. One station houses the cancer stock and the other the normal stock. The latter has been inbred for the past 2 years, no outside accessions having been made during this period, and is employed in the general investigative work of the Institute. The completeness with which separation between the two stations has been effected is indicated by the fact that in the long period during which deaths—many or few—from mouse

[2] Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25.

[3] Amoss, H. L., and Haselbauer, P. P., *J. Exp. Med.*, 1922, xxxvi, 107.

typhoid were taking place among the cancer stock there were only four deaths among the normal stock apparently from this cause. The population of the normal stock was kept approximately at from 2,500 to 3,000 individuals.

The Epidemic.

The course of the epidemic outbreak can best be followed by observing the graphic curve which is reproduced in two sections which have been subdivided into segments (Text-fig. 1).

As the first segment (No. 1) of the curve indicates, the death rate among the mice rose in the 2nd week of September and actually became about twice the usual weekly rate. From this time on the records were kept more accurately than before and they show a gradual rise in the number of deaths up to the 1st week in November, when the rate remained constant for 10 days, increased during the 3rd week, and began to decline abruptly.

The curve indicating actual number of deaths forms a plateau extending over a period of 2 weeks during which there was a slight depression. The decline was slightly more irregular than the rise.

The course of the epidemic is perhaps better described by the attack rate per 1,000 of population, which rose sharply until November 19 and declined as rapidly as it had risen.

The return to what is regarded as the usual death rate came about January 18, 1919. The outbreak lasted about 140 days; the peak was reached at about the middle of this period, or the 80th day (November 19), so that the curve representing the rate per 1,000 is fairly symmetrical with a slight lag in the decline.

From a consideration of the character of the curve during December, there is a suggestion of the occurrence of another small wave which might have begun somewhere between December 15 and 20 and reached its peak on December 29. The interval between the peaks is about 15 days. Such a supposition is strengthened by the occurrence of a similar wave in March (shown in Segment 2) which endured for 15 days. It is not improbable that the total outbreak is really a series of six overlapping epidemic waves, each lasting about 15 days. Unfortunately no spot map was kept recording the distribution of the deaths in the breeding station. In general the epi-

TEXT-FIG. 1. Segment 1. Deaths from all causes among the cancer breeding stock by 5 day periods from September 5, 1918, to January 18, 1919.

Segment 2. Deaths from all causes by 5 day periods from January 19 to April 30, 1919.

Segment 3. Deaths from all causes by 5 day periods from May 1 to September 30, 1919.

Segment 4. Deaths from all causes by 5 day periods from October 1, 1919, to February 29, 1920.

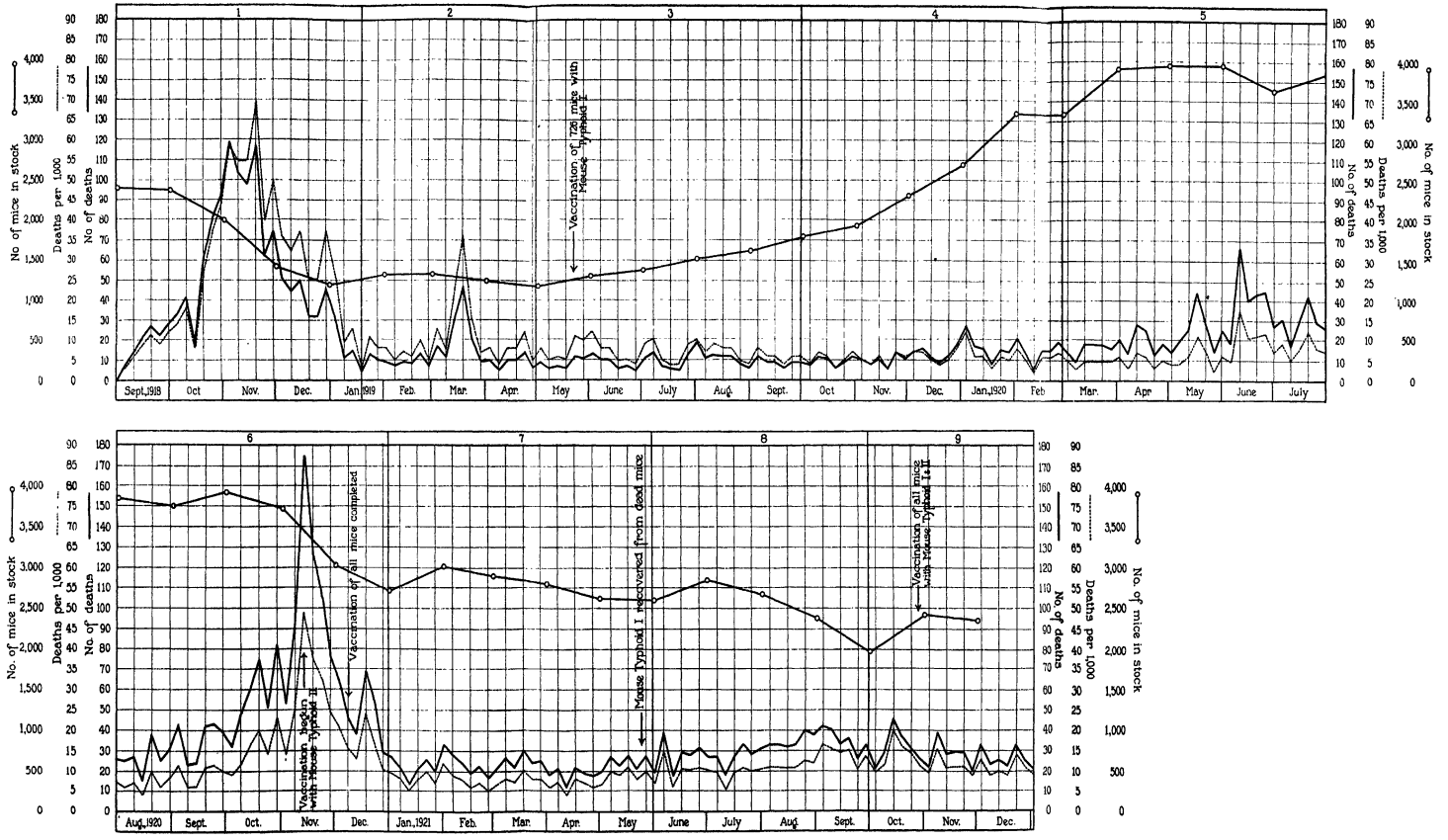
Segment 5. Deaths from all causes by 5 day periods from March 1 to July 31, 1920.

Segment 6. Deaths from all causes by 5 day periods from August 1 to December 31, 1920.

Segment 7. Deaths from all causes by 5 day periods from January 1 to May 31, 1921.

Segment 8. Deaths from all causes by 5 day periods from June 1 to September 30, 1921.

Segment 9. Deaths from all causes by 5 day periods from October 1 to December 31, 1921.



TEXT-FIG. 1. ----------Deaths per 1,000 of mouse population by 5 day periods. ——— Actual number of deaths in mouse population by 5 day periods. ——— Total number of mice in stock according to the monthly census.

demic raged on the shelves where the older mice were kept. For example, among 1,465 older mice the mortality was 82 per cent, whereas among 1,034 new mice, most of which were weaned before the onset of the epidemic, the mortality was 43 per cent.

The severity of this first, or as it may be called November epidemic wave, is shown by the number of deaths presumably in all instances from mouse typhoid. This number as calculated for the period from September 10 to January 28 inclusive is 1,351 mice, or a little more than one-half of the population of the breeding station. During December the deaths occurred mainly in boxes which had already been attacked. Instances of spread to new boxes were frequent.

It is quite certain that this large number of deaths was brought about by the mouse typhoid infection, for while not every dead mouse was studied bacteriologically, yet all were submitted to autopsy and the usually obvious signs of the disease detected.

Along with the epidemic there occurred as a concomitant effect a large reduction in the birth rate so that the number of mice diminished through death was not augmented by births. Hence during the next period of January, February, and 1st week in March, as shown by the second segment (No. 2) the death rate, while still high as compared with the pre-epidemic period, kept quite uniform. However, during the 1st week of March an upward tendency is evident, which at the beginning of the 2nd week is converted into a steady rise culminating in a peak lower than the November peak, enduring a shorter time, and falling more sharply, as the average death rate is again reached in the 1st week of April.

This second, or March epidemic wave is to be considered in the light of two classes, as it were, of the mouse population; namely, the old population which had passed through the November epidemic, and the accessions through new births. The total population had been profoundly reduced by the November epidemic and not yet restored by new births. The March wave was of short duration (15 to 20 days) and was approximately half as severe as that of November. As already stated, the November outbreak may be looked upon as a series of waves following closely one another, whereas the March epidemic represented a single wave.

A period of relative quiescence now set in, as shown by Segment 3. On May 17 the total mouse population was about 1,250 individuals. During the next 4 weeks, half of the mice were given (vaccinated) under the skin of the back a single injection of 0.2 cc. of a suspension (or about 600,000) of killed bacilli of the strain of mouse typhoid isolated during the epidemic. The vaccination produced no immediate effect on the death rate, although it is noteworthy that during a 4 weeks period of mid-May to mid-June, only two of the vaccinated mice died and in only one was the mouse typhoid bacillus found.

The carrying out of the vaccination would seem rather to have coincided with than to have been responsible for the quiescent period which extended from June, 1919, to about January, 1920, the level of which is shown in Segments 3 and 4. During this period the mouse population was rising steadily chiefly through new births. The new individuals were left with the old and no further vaccination was carried out in the breeding station. Although the general death rate had not fallen to that of the pre-epidemic period, yet the new equilibrium which had been established was regarded as satisfactory.

However, beginning in January, 1920, as the population continued to increase, a small rise in death rate also occurred as is shown in Segments 4 and 5. By May, 1920, the total population in the breeding station had reached approximately 4,000. As presaging of the next epidemic, events may be detected in the rise in number of deaths in April, May, and June, as indicated in Segment 5. Although the population had again become large, this mere numerical increase was followed with some anxiety.

The number of deaths per 1,000 continued generally high during July, August, and September. Beginning the 1st week in October, a further rise took place that with minor and perhaps unimportant fluctuations led into the sharp epidemic wave of November, 1920, which exceeded in actual number of deaths that of the first, or 1918 November wave, but the rate per 1,000 was only five-sevenths of that recorded in the 1918 outbreak. The course of this epidemic wave which was practically obliterated by January, 1921, is shown in Segment 6. The wave-like character of the curve is also present. Whereas the epidemic of 1918 seemed to be a summation of six waves, the 1920 outbreak consists of only four, each of which was longer and lower than in the former.

An analysis of this epidemic wave yields the following data. The total mouse population affected by this epidemic wave during its entire course was 4,282, of which 1,463 died. Hence the gross mortality was 34 per cent. Of the 4,282 mice in the total population, 220 were at least 18 months old and had thus passed through the previous severe epidemic wave, while 4,062 were new individuals not so severely exposed. Taking the two classes of the population separately, the old mice (18 months old at least),[4] and the new mice as above described, it may be stated that of 220 of the former, 130, or 60 per cent, succumbed in the November, 1920, epidemic wave, while of 4,062 of the latter, or new stock, only 1,333, or 32.8 per cent, fell victims. The effect of the epidemic on the birth rate was marked. During the extreme height and the following wave in November and December, very few births took place. By January 1, 1921, the total population had fallen to 2,731 and the sharp outbreak may be regarded as having come to an end. For the succeeding 5 months (January to May) the death rate remained at the average low level ordinarily observed in stocks of mice (Segment 7). The usual[1] slight increase in death rate was observed during the summer (Segment 8) and autumn (Segment 9).

There is practically no difference in the seasonal distribution of the two major outbreaks. Each was preceded by a slightly increased death rate during the summer months and began to gain headway in September, reaching the peak on November 19, 1918, and November 13, 1920, respectively. Since the death rate is calculated on a basis of 5 day periods, the difference of 6 days is probably within the limit of error. The entire duration of the 1918 outbreak was about 140 days and of the 1920 epidemic 125 days.

The bacteriology of the November, 1920, outbreak has particular importance. Practically all the dead mice were examined post-mortem, and cultures of the mouse typhoid bacillus were obtained by Mr. Sturm from approximately 75 per cent of the examined animals. Of the many cultures thus obtained, two strains were retained and eventually turned over to Dr. Amoss for use in his experiments.

[4] The average longevity of mice in non-infected stock is about 2 years. Old age must be considered as contributory to a high death rate in such a group.

The immunological study by Dr. Amoss of these cultures showed that they differed from the original cultures isolated in the 1918 epidemic. A full description of this bacillus will be found in another paper of this series.[3]

The fact that the two strains of the paratyphoid-enteritidis group, differing immunologically from each other and both potentially capable of setting up severe epidemics among mice, were responsible for the epidemics separated from each other by 2 years, comes to have a special interest and may possess a particular significance in view of the vaccinations carried out in the period between the two epidemics. For superficially, at least, it appears that the inoculation of the killed cultures of the first bacillus shunted, as it were, that particular organism out of action while leaving the recruited population, both old and new, and the old even more than the new, subject to a second variety of the mouse typhoid bacillus. If this is at all a true statement of what has taken place in the second November epidemic, then the vaccination of part of the surviving population in May and June, 1919, was sufficient to protect the entire population from infection with the first variety of the mouse typhoid bacillus. This last point is obviously one that is open to experimental inquiry as is also the collateral point whether cross-immunity reactions occur between the two varieties of the *Bacillus enteritidis* with which we have been dealing.

No further outbreak of mouse typhoid of an epidemic character has taken place in the cancer breeding station since January, 1921. Just as a large part of the surviving mice was vaccinated after the November, 1918, epidemic wave, so vaccination of all the survivors was carried out at the height of the November, 1920, epidemic wave. The vaccine employed in 1920 was identical with that employed in 1919, and as the two varieties of inciting bacilli differ immunologically, it may be regarded as at least questionable whether the second inoculations have had anything to do with the relative quiescence of the mouse typhoid infection in the breeding station following the last November epidemic.

A slight increase in the death rate occurred during the summer of 1921, and since Mouse Typhoid I had been recovered from some of the mice dying in May the entire population was vaccinated with Mouse Typhoid I and II in October.

Carriers.

No extensive study was made of carriers. In January, 1922, ten mice which had passed through the second epidemic and ten mice born of these were killed and cultured. The cultures were negative for Mouse Typhoid I and Mouse Typhoid II, except for those from one mouse in the former group. In this mouse Mouse Typhoid I was recovered from the cecum only. Cultures from the small intestine were negative for Mouse Typhoid I and Mouse Typhoid II.

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PHOSPHORIC ESTERS OF SOME SUBSTITUTED GLUCOSES AND THEIR RATE OF HYDROLYSIS.

BY P. A. LEVENE AND G. M. MEYER.

WITH THE ASSISTANCE OF I. WEBER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 24, 1922.)

A previous communication contained a report[1] on the constants of hydrolysis of several phosphoric acid esters of glucose. The allocation of the phosphoric acid radical was based on the work of Irvine and Scott[2] on the structure of diacetone glucose. At that time we had overlooked a later publication from Irvine's laboratory in which the previous theory of the structure of diacetone glucose was revised. The positions therein assigned by Macdonald[3] to the acetones are 1, 2- and 5, 6-, hence the benzoyl derivative obtained from it is 1,2-5,6- diacetone-3-benzoyl glucose.

On this basis, the two phosphoric acid esters, one derived from the diacetone glucose, the other from the diacetone monobenzoyl derivative have the following structure: the former 1,2- 5,6-diacetone-3-phosphoric acid glucose and the latter 1,2-acetone-3-benzoyl-5- or 6-phosphoric acid glucose.

It seems a strange coincidence that Karrer and Hurwitz[4] also overlooked the work of Macdonald and recently published a paper in which they dealt with the structure of diacetone glucose and assigned to it the same structure which had been formulated by Macdonald.

According to the present formulation the more resistant compound is the one in which the phosphoric acid radical is in position 5 or 6, probably in position 6. This is in harmony with the previous observations on the rate of hydrolysis of 5-phosphoric acid ribose. The latter was found very resistant to hydrolytic agents.

[1] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1921, xlviii, 233.

[2] Irvine, J. C., and Scott, J. P., *J. Chem. Soc.*, 1913, ciii, 568.

[3] Macdonald, J. L. A., *J. Chem. Soc.*, 1913, ciii, 1896.

[4] Karrer, P., and Hurwitz, O., *Helvetica Chim. Acta*, 1921, iv, 728.

At the time when the allocation 1,2-3,5 was assigned to the acetones in the diacetone glucose the position 5,6 was assigned to the benzaldehyde in benzylidene glucoside. Hence the respective positions of the inorganic acid in the phosphoric esters of the two sugar derivative should be different. These considerations led us to prepare the substance.

On the basis of the latest formulation the second acetone in the diacetone glucose and the benzaldehyde in the benzylidene monoacetone glucose should each enter into positions 5, 6. Hence the phosphoric acid derivative of diacetone glucose and that of 1,2-acetone and 5,6-benzylidene glucose should both have the phosphoric acid in position 3. To our surprise the constant of hydrolysis of the phosphoric ester obtained from the benzylidene derivative was found $17(10^{-3})$, which is of the same order of magnitude as that of the phosphoric acid derivative obtained from 1,2-acetone-3-benzoyl glucose, whereas a constant of about $54(10^{-3})$ was expected.

A simple explanation for this unexpected result may be found in the assumption that in the process of preparation the benzaldehyde is cleaved off before the phosphoric acid combines with the glucose. This possibility requires special consideration in view of the fact that the product obtained from benzylidene acetone glucose was acetone phosphoric acid glucose. Thus the benzaldehyde group was lost in the process of preparation. There is reason to believe that the cleavage of the benzaldehyde took place after the union with phosphoric acid was accomplished. Had the cleavage of the benzaldehyde been the first step in the operation, then the resulting substance would be 1,2-monoacetone glucose; however, when this compound is acted upon by phosphorous oxychloride a product is obtained which has a constant of hydrolysis = $44(10^{-3})$. Furthermore, when diacetone glucose is treated with phosphorous oxychloride two esters are obtained, one of diacetone glucose and the second of monoacetone glucose. The constants of hydrolysis of both are practically identical, namely $56(10^{-3})$ and $58(10^{-3})$, thus showing that the position of the phosphoric acid in both is identical and hence indicating that the cleavage of the acetone took place after the phosphoric acid had united with the glucose.

A priori two other alternative explanations are possible. One is that the position of the benzaldehyde in the benzylidene acetone glucose is not on carbon atoms 5 and 6, the second that in the course of the reaction the phosphoric acid radical wanders from position 3 to either 5 or 6. Further investigations will deal with this problem.

The findings on the phosphoric esters of the methylated sugars are also now seen in a different light. The abnormal course of the hydrolysis of 2-phosphoric acid-3,5,6-trimethyl-methyl glucoside is apparently due to the fact that the substance contained a very small admixture of 6-phosphoric acid -2, 3, 5-trimethyl-methyl glucoside. The hydrolysis of the principal substance was undoubtedly completed in the first 60 minutes. Its constant of hydrolysis is not less than $84(10^{-3})$ or $87(10^{-3})$.

The following table contains the rates of hydrolysis of the phosphoric esters of substituted glucoses with special reference to the allocation of the phosphoric acid.

		K
1	5- or 6 (?)-phosphoric acid methyl glucoside (from α-methyl glucoside)	$22(10^{-3})$
2	Mixture of 3-phosphoric acid with 5- or 6-phosphoric acid monoacetone glucose (from monoacetone glucose)	$44(10^{-3})$
3	3-phosphoric acid-1, 2- 5, 6-diacetone glucose (from diacetone glucose)	$56(10^{-3})$
4	3-phosphoric acid-1, 2-monoacetone glucose (by-product from diacetone glucose)	$58(10^{-3})$
5	5- or 6-phosphoric acid-3-monobenzoyl-1, 2-acetone glucose (from 1, 2-monoacetone-3-monobenzoyl glucose)	$18(10^{-3})$
6	5- or 6-phosphoric acid-1, 2-acetone glucose (from 5 by removing benzoyl group)	$24(10^{-3})$
7	5- or 6 (?)-phosphoric acid-1, 2-acetone glucose (from benzylidene monoacetone glucose)	$17(10^{-3})$
8	6-phosphoric acid-2, 3, 5-trimethyl-methyl glucoside	$44(10^{-3})$
9	2-phosphoric acid-3, 5, 6-trimethyl-methyl glucoside	$87(10^{-3})$

EXPERIMENTAL.

Benzylidene Monoacetone Glucose.

50 gm. of monoacetone glucose are heated with 300 cc. of freshly distilled benzaldehyde and 50 gm. of anhydrous sodium sulfate at

145°C. for 5 hours. While still warm, the solution is filtered into a distilling flask and the larger part of the benzaldehyde removed by distillation under diminished pressure. When the contents of the flask show a tendency to gelatinize, they are poured into 1 liter of ligroin (80–90°). On vigorous stirring the oily mass soon crystallizes. The crude product is filtered and washed with cold dry ether, in which it is practically insoluble. A nearly pure white product is obtained. Recrystallized several times from absolute alcohol this melts at 141–142°C. The yield is 20 gm.

0.1008 gm. substance: 0.2306 CO_2 and 0.0628 gm. H_2O.

$C_{16}H_{20}O_6$. Calculated. C 62.3, H 6.48.
Found. " 62.38, " 6.97.

The substance had the following rotation:

$$[\alpha]_D^{15} = \frac{+0.44° \times 100}{2 \times 1} = +22°.$$

1, 2-Monoacetone-6-Phosphoric Acid Glucose from Benzylidene Monoacetone Glucose.

20 gm. of dried benzylidene monoacetone glucose are dissolved in 75 cc. of dry pyridine and cooled to $-20°$C. To this is added at once, a solution of 10 gm. of phosphorous oxychloride in 25 cc. of pyridine. The temperature of the reaction mixture does not go above $+20°$C. and crystals of pyridine hydrochloride settle out immediately on cooling. If the oxychloride solution is added too slowly the reaction is incomplete and only a small quantity of pyridine hydrochloride separates. After allowing the reaction mixture to stand for 1 hour, cold water is added. After the addition of 120 gm. of barium hydroxide, the pyridine is removed by distillation under diminished pressure. The residue is then neutralized wth sulfuric acid until just acid to Congo red, shaken for ½ hour with 60 gm. of silver sulfate, and filtered. The filtrate is treated with hydrogen sulfide, the resulting precipitate removed by filtration. After removing the hydrogen sulfide by a current of air the solution is again made alkaline with barium hydroxide. The excess barium is removed by means of carbon dioxide and the filtrate concentrated under diminished pressure

to a syrup. This is taken up in absolute alcohol, filtered, and the barium salt precipitated in a large volume of dry ether. The substance analyzed for a monoacetone phosphoric acid glucose.

0.1104 gm. substance: 0.1156 gm. CO_2, 0.042 gm. H_2O, and 0.0481 gm. ash.
0.2916 " " : 0.0746 " $Mg_2P_2O_7$
0.0972 " " : 0.0400 " $BaSO_4$.

$C_9H_{16}O_6H_2PO_3$. Calculated. C 35.75, H 6.00, P 11.28.
Found (calculated Ba-free). " 37.7, " 5.46, " 9.45.

Monoacetone Phosphoric Acid Glucose from Monoacetone Benzylidene Glucose.

7.271 gm. of the barium salt of this substance were dissolved in water and the volume was made up to 50 cc. Of this solution 3 cc., equivalent to 0.03 gm. of P, were put into glass tubes together with 2.1 cc. of N H_2SO_4 and 0.9 cc. of water and sealed. The tubes were heated at 100°C. for the intervals indicated in the following table. The contents of each tube were made up to 100 cc. and the P in 40 cc. portions was determined.

5-6 (?)-Phosphoric Acid-1, 2-Acetone Glucose.

Time.	$Mg_2P_2O_7$ in 40 cc.	Average.	P in $Mg_2P_2O_7$ in 100 cc.	P in free acid.	P of total P.	x	$a - x$	K
min.				*per cent*	*per cent*	*gm. $Mg_2P_2O_7$*	*gm. $Mg_2P_2O_7$*	$\frac{1}{t}\log\frac{a}{a-x}$
120	0.0018	0.0020	0.0014	0.43	4.6	0.0050	0.1027	0.000176
	0.0022							
240	0.0044	0.0030	0.0030	0.99	10.0	0.0107	0.0970	0.000182
	0.0042							
360	0.0060	0.0055	0.0038	1.19	12.8	0.0138	0.0939	0.000166
	0.0050							
480	0.0072		0.0050	1.57	16.8	0.0181	0.0896	0.000167

$a = 0.1077$

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SULFURIC ESTERS OF SOME SUBSTITUTED GLUCOSES AND THEIR RATE OF HYDROLYSIS.

BY P. A. LEVENE AND G. M. MEYER.

WITH THE ASSISTANCE OF I. WEBER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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It has been demonstrated in this laboratory that all known glucoproteins are protein derivatives of complex substances which are essentially sulfuric acid esters of disaccharides. It has been observed that individual esters of this group differ in their stability. In some of them the sulfuric acid is removed from the disaccharide very readily, whereas in other compounds it is removed with greater difficulty. *A priori* it seemed possible to explain the differences in stability of individual compounds by the difference in the position of the sulfuric acid on the glucose molecule. In this respect the behavior of the conjugated sulfuric acids resembles that of the conjugated phosphoric acids. In regard to the phosphoric esters of glucose,[1] it was demonstrated experimentally that their resistance is determined by the position of the phosphoric acid on the sugar molecule.

These considerations led us to synthesize two sulfuric acid sugar derivatives, one from diacetone glucose and the other from 1, 2-acetone-3-benzoyl glucose. The first should yield a substance with the sulfuric acid attached to carbon atom 3, the second to either carbon atom 5 or 6.

For the preparation of sulfuric acid esters of glucose several methods have been recommended by Neuberg and Pollak,[2] and Neuberg and Liebermann.[3] They employed for the purpose either pyrosulfates or chlorosulfonic acid. Neuberg and Pollak have also mentioned that sulfuryl chloride may be used for this purpose. However, for the

[1] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1922, liii, 431.

[2] Neuberg, C., and Pollak, H., *Biochem. Z.*, 1910, xxvi, 515.

[3] Neuberg, C., and Liebermann, L., *Biochem. Z.*, 1921, cxxi, 326.

use of this reagent no experimental directions are given by them. Helferich[4] has also worked with sulfuryl chloride and obtained a dichlorohydrinsulfate. In the present experiments sulfuryl chloride was employed. The success of the synthesis depends largely on the temperature maintained during the operation. It was found advantageous to add the solution of sulfuryl chloride in chloroform to a pyridine solution of the sugar derivative cooled to −10°C., and to allow the temperature to rise to about 30°C. The details of the preparation are given in the experimental part.

Regarding the rates of hydrolysis it was found that the ester having the sulfuric acid in position 5 or 6 was more stable than the one having the acid in position 3 as may be seen from the following table.

	$K = \frac{1}{t} \log \frac{a}{a-x}$
3-sulfuric acid-1, 2- 5, 6-diacetone glucose	.60 (10^{-3})
5- or 6-sulfuric acid-1, 2-acetone glucose	.40 (10^{-3})

This is in harmony with the views expressed in the publications from this laboratory on the structure of chondroitin and mucoitin sulfuric acids.

EXPERIMENTAL.

Sulfuric Acid Diacetone Glucose.

A solution of 10 gm. of diacetone glucose in 30 cc. of dry pyridine, is cooled to − 10°C. To this is added a solution of 2.7 cc. of sulfuryl chloride in 25 cc. of dry chloroform, also cooled to − 10°C. The temperature rises to 30°C., and the reaction product is allowed to stand at room temperature over night. The product is then dark red and contains no precipitate. Water and 60 gm. of barium hydroxide are then added and the chloroform and pyridine removed by distillation under diminished pressure. The solution is treated with sulfuric acid until it turns acid to Congo red and is shaken for ½ hour with 30 gm. of silver sulfate. The excess silver is removed by hydrogen sulfide, which is then removed by a current of air. The excess of barium is removed by means of carbon dioxide and the filtrate is concentrated to dryness under diminished pressure. The residue is

[4] Helferich, B., *Ber. chem. Ges.*, 1921, liv, 1082.

dissolved in a small amount of absolute alcohol, the solution is filtered and precipitated in a large volume of dry ether.

For purification the precipitate is redissolved in absolute alcohol and reprecipitated in ether, this process is repeated several times.

10 gm. of diacetone glucose yield 5 gm. of the barium salt of sulfuric acid diacetone glucose.

The substance does not reduce Fehling's solution until after hydrolysis. Barium chloride, also, produces no precipitate in an aqueous solution of the substance. However, a precipitate of barium sulfate is formed after the substance is hydrolyzed with hydrochloric acid.

The elementary composition of the substance is not so good as is desired. However, taking into consideration the amorphous nature of the substances and their great solubility, the result may be regarded as satisfactory.

0.1096 gm. substance: 0.1334 gm. CO_2 and 0.0454 gm. H_2O.
0.2766 " " : 0.1320 " $BaSO_4$ (S determination).
0.0922 " " : 0.0254 " " (Ba ").

$(C_{12}H_{20}O_9S)_2Ba$. Calculated. C 35.5, H 4.66, S 7.88, Ba 16.85.
Found. " 33.19, " 4.63, " 6.55, " 16.21.

5- or 6 (?)-Sulfuric Acid-Monoacetone Glucose from 3-Benzoyl-1, 2-Monoacetone Glucose.

Benzoyl monoacetone glucose (10 gm.) dissolved in 30 cc. of dry pyridine was reacted on with 2.7 cc. of sulfuryl chloride dissolved in 25 cc. of chloroform, as previously described under sulfuric acid diacetone.

The final residue was dissolved in a small quantity of hot absolute alcohol. On cooling barium benzoate crystallized. This was filtered off and the filtrate precipitated in a large volume of dry ether. This process was repeated several times.

The substance obtained analyzed for sulfuric acid monoacetone. It does not reduce Fehling's solution. An aqueous solution of the substance gives no precipitate with barium chloride until after hydrolysis with acid.

0.1062 gm. substance: 0.1136 gm. CO_2, 0.0352 gm. H_2O, and 40.3 gm. ash.
0.2912 " " : 0.1534 " $BaSO_4$ (S determination).
0.0971 " " : 0.0390 " " (Ba ").

$(C_9H_{15}O_9S)_2Ba$. Calculated. C 29.4, H 4.3, S 8.7, Ba 18.35.
Found. " 29.17 " 3.7, " 7.23, " 23.63.

Sulfuric Acid Diacetone Glucose.

3.817 gm. of the barium salt of this substance were dissolved in a little water and the volume was made up to 25 cc. Of this solution 3 cc. equivalent to 0.031 gm. of S, were put into glass tubes together with 1.68 cc. of N HCl and 4.32 cc. of H_2O. After the tubes were sealed they were heated at 75°C. for the time intervals indicated in the following table. The sulfuric acid was determined as $BaSO_4$.

Time.	$BaSO_4$	S in $BaSO_4$.	S in free acid.	S of total S.	x	$a-x$	K
min.	*gm.*	*gm.*	*per cent*	*per cent*	*gm. $BaSO_4$*	*gm. $BaSO_4$*	$\frac{1}{t}\log\frac{a}{a-x}$
60	0.0153	0.0021	0.547	7.00	0.0153	0.2032	0.00053
240	0.0620	0.0085	2.22	28.4	0.0620	0.1565	0.00060
360	0.0857	0.0118	3.07	39.2	0.0857	0.1328	0.00060

$a = 0.2184$

Sulfuric Acid Monoacetone Glucose.

3.481 gm. of the barium salt of this substance were dissolved in a little water and the volume was made up to 25 cc. Of this solution 3 cc., equivalent to 0.031 gm. of S, were put into glass tubes together with 1.94 cc. of N HCl and 4.06 cc. of H_2O. After the tubes were sealed they were heated at 75°C. for the time intervals indicated in the following table. The sulfuric acid was determined as $BaSO_4$.

Time.	$BaSO_4$	S in $BaSO_4$.	S in free acid.	S of total S.	x	$a-x$	K
min.	*gm.*	*gm.*	*per cent*	*per cent*	*gm. $BaSO_4$*	*gm. $BaSO_4$*	$\frac{1}{t}\log\frac{a}{a-x}$
120	0.0119	0.0016	0.502	5.45	0.0119	0.2066	0.00040
240	0.0480	0.0066	2.02	22.0	0.0480	0.1705	0.00044
360	0.0622	0.0085	2.62	28.5	0.0622	0.1563	0.00040

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PREPARATION AND ANALYSIS OF ANIMAL NUCLEIC ACID.

BY P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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The preparation of a pure animal nucleic acid remained a difficult task notwithstanding the fact that several methods have been recommended in recent years. A year ago the present writer made an effort to prepare animal nucleic acid by his picric acid method, slightly modifying the details of the procedure. The result was quite satisfactory when small quantities of the material were prepared by the writer personally. However, when the preparation of the material was left in the hands of technical assistants the product obtained by them was rather impure and besides had a brownish color which made the measurement of its optical activity very difficult. A new effort was made to work out an easy and reliable process. Preliminary to this attempt we tested all the recently published methods of W. Jones,[1] R. Feulgen,[2] and E. J. Baumann.[3] None proved satisfactory, since every sample prepared by any one of these methods gave a very marked biuret reaction. That the authors themselves obtained satisfactory results by their respective methods is not doubted, but we affirm that such results, to say the least, are not constant. Material prepared by the method of Jones[1] by a French firm also proved unsatisfactory.

After several trials we finally modified our older method by substituting colloidal iron for the picric acid.

The details of the process as carried out at present are as follows: The glands (10 lbs.) are dissected free from fat, ground in a chopping

[1] Jones, W., Nucleic acids; their chemical properties and physiological conduct, New York, 2nd edition, 1920.

[2] Feulgen, R., *Z. physiol. Chem.*, 1917–18, ci, 296.

[3] Baumann, E. J., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 118.

machine, and transferred into 5 liters of water, containing 250 gm. of sodium hydroxide. The mixture is boiled for 35 minutes and then neutralized with acetic acid. 50 cc. of a colloidal iron solution (iron dialyzed, 5 per cent Fe_2O_3, Merck) are added and the solution is filtered and allowed to stand over night. To the filtrate is then added a double volume of methyl alcohol containing 2 per cent of hydrochloric acid. The precipitate thus formed is filtered off and washed with methyl alcohol until the filtrate is free from hydrochloric acid.

This process has been applied to the preparation of nucleic acid from thymus gland, spleen, kidney, pancreas, and liver. The average respective yields from 10 lbs. of gland were: thymus, 150.0 gm.; spleen, 40.0 gm.; kidney, 25.0 gm.; pancreas, 35.0 gm.; and liver, 18.0 gm.

From the first four organs the first precipitation yields a material which is either entirely free, or contains barely detectable traces, of biuret-giving substances. In the last case one reprecipitation with alcohol containing 2 per cent hydrochloric acid suffices to purify the material. The material obtained from the liver contains a marked proportion of glycogen. Such preparations are readily purified by dissolving them in water with the aid of a minimum amount of alkali, slightly acidulating with hydrochloric acid, and precipitating the nucleic acid with a 20 per cent solution of cupric chloride. The precipitate is washed with water, suspended in alcohol containing 2 per cent hydrochloric acid, and carefully triturated until the larger part of the copper is recovered. The precipitate is then dissolved in water, adding a minimum amount of alkali, and the nucleic acid is precipitated by a double volume of alcohol containing 2 per cent hydrochloric acid. When this process fails, the crude nucleic acid may be dissolved in water with the aid of a minimum amount of alkali and precipitated with hydrochloric acid. This precipitate is then redissolved in water, by the addition of a minimum amount of alkali and to the solution an equal volume of alcohol, containing 2 per cent of hydrochloric acid, is added.

The following table contains the results of the analysis of various samples obtained by this process:

	C	H	N	P
Thymus gland	36.72	4.58	14.59	9.05
Spleen	36.21	4.30	14.54	8.94
Kidney	36.51	4.17	14.07	8.74
Pancreas	36.34	4.40	14.37	9.10
Liver	35.69	4.05	15.25	9.05
Calculated for a hexose tetranucleotide	36.30	4.19	14.79	8.73

The agreement of the analytical results with the theory is quite satisfactory. However, not too much importance should be attached to it, since the elementary composition of amorphous substance has only a relative theoretical importance. Besides, not all samples gave identical analytical results. Often the original material contained 10 per cent of phosphorus, and about 12 per cent of nitrogen, but showed little deviation from the theory in the content of carbon and hydrogen. On the other hand, the table is very important in showing that nucleic acids derived from different organs do not vary in their elementary composition notwithstanding the claims to the contrary recently expressed by Feulgen and others.

Also as regards the content of purine bases, the acids from the various organs seem to show only such variations as could be expected from the degree of accuracy of the analytical methods. Thus the theory of the tetranucleotide requires for adenine picrate 27.10 per cent and for guanine 10.6 per cent purine bases.

The results of the analysis were as follows:

	Adenine picrate.	Guanine.
Thymus gland	26.6	11.8
Spleen	26.6	13.0
Kidney	23.3	12.6
Pancreas	23.0	11.5
Liver	30.0	11.8

ANALYTICAL PART.

The hydrolysis of the nucleic acids for the purpose of estimating the purine bases was carried out in the same way as described in a previous article, with the exception that instead of absolute methyl alcohol,

one containing 5 per cent of water was employed. The nucleic acid (50 gm.) is suspended in 500 cc. of 95 per cent methyl alcohol and hydrogen chloride gas is passed for 2 hours. The acid soon dissolves and gradually the hydrochlorides of the bases settle out. To complete the separation the reaction product is allowed to stand over night.

Separation and Purification of the Purine Bases.—After a scrutiny of the analytical data on the nucleic acids published by different writers one is left with the impression that the workers encountered difficulties in obtaining the bases in analytically pure condition. This is particularly true regarding guanine. In part this difficulty arose from the method of hydrolysis, in part also from the method of isolating the bases. It is possible, however, to prepare the bases in pure condition in a very short time. The process employed in the course of this work was the following:

1. Separation of Adenine from Guanine.—The hydrochlorides are dissolved in hot water and the solution is neutralized with sodium hydroxide until neutral to Congo red. The guanine then precipitated out. A precipitate is then formed which consists practically of pure guanine, slightly contaminated with adenine. In order to remove this it is again dissolved in dilute hydrochloric acid and again precipitated with sodium hydroxide. The two filtrates are combined and the adenine is precipitated in the form of the picrate. In the course of the present work the crude adenine picrate and the crude guanine were dried to constant weight. The crude adenine picrate contains about 28 per cent of nitrogen whereas 29.3 per cent is required by theory and the guanine contained about 40 per cent of nitrogen whereas 46.35 per cent is required by theory.

2. Purification of the Base.—Adenine picrate is obtained analytically pure by one recrystallization from 25 per cent solution of acetic acid. The crude picrate (10 to 12 gm.) is suspended in 1 liter of the acid, and boiled until dissolved.

Guanine is obtained analytically pure in the following manner. The crude material is dissolved in boiling dilute sulfuric acid and precipitated by means of silver sulfate. The precipitate of guanine silver sulfate is filtered off while the reaction product is still hot. The silver salt is decomposed by means of hydrochloric acid and the clear filtrate

from silver chloride is neutralized with 10 per cent of sodium hydroxide. Free guanine is thus precipitated. On some occasions it was found necessary to repeat the silver precipitation process.

Analysis of Individual Nucleic Acids.

Thymus Gland.

0.1184 gm. substance: 0.1384 gm. CO_2 and 0.0420 gm. H_2O.
0.1736 " " required (Kjeldahl) 18.10 cc. 0.1 N acid.
0.2605 " " : 0.0846 gm. $Mg_2P_2O_7$.
Found. C 36.72, H 4.58, N 14.59, P 9.05.

Bases.—45.0 gm. of the dry material yield 12.0 gm. (26.6 per cent) of crude adenine picrate and 5.3 gm. (11.8 per cent) of crude guanine, containing 40 per cent nitrogen.

Adenine picrate analyzed as follows:

0.1000 gm. required (Kjeldahl, reduction with zinc) 20.75 cc. 0.1 N acid.
$C_5H_5N_5C_6H_2(NO_2)_3OH + H_2O$. Calculated. N 29.31.
Found. " 29.05.

Guanine was analyzed as the free base.

0.0983 gm. substance required (Kjeldahl) 32.25 cc. 0.1 N acid.
$C_5H_5N_5O$. Calculated. N 46.35.
Found. " 45.93.

Spleen Nucleic Acid.

0.1006 gm. substance: 0.1444 gm. CO_2 and 0.0418 gm. H_2O.
0.1795 " " required (Kjeldahl) 18.65 cc. 0.1 N acid.
0.2693 " " : 0.0864 gm. $Mg_2P_2O_7$
Found. C 36.21, H 4.30, N 14.54, P 8.94.

Bases.—45.0 gm. of the dry acid gave 12.0 gm. (26 per cent) of crude adenine picrate and 6.0 gm. (13.3 per cent) of crude guanine, having 39.2 per cent of nitrogen.

Adenine was purified by recrystallization and analyzed as follows:

0.1000 gm. substance: (Dumas) 26 cc. nitrogen at 26°C., 756 mm.
$C_5H_5N_5$. $C_6H_2(NO_2)_3OH + H_2O$. Calculated. N 29.31.
Found. " 29.51.

Guanine was analyzed both as the free base and as the picrate.

0.0988 gm. substance required (Kjeldahl) 32.35 cc. 0.1 N acid.
$C_5H_5N_5O$. Calculated. N 46.35.
Found. " 45.84.

This substance was dissolved in water on addition of the required amount of 10 per cent sodium hydroxide. An excess of picric acid was then added. The picrate settled out gradually in the form of long needles and analyzed as follows:

0.1000 gm. substance: (Dumas) 24.2 cc. nitrogen at 23°C., 763 mm.
$C_5H_5N_5O$. $C_6H_2(NO_2)_3OH+H_2O$. Calculated. N 28.13.
Found. " 28.02.

Kidney Nucleic Acid.

0.0992 gm. substance: 0.1312 gm. CO_2 and 0.0378 gm. H_2O.
0.1693 " " required (Kjeldahl) 16.95 cc. 0.1 N acid.
0.2539 " " : 0.0834 gm. $Mg_2P_2O_7$.
Found. C 36.06, H 4.26, N 14.01, P 9.15.

Bases.—42.0 gm. of the substance yielded 9.8 gm. (23.3 per cent) of adenine picrate and 5.3 gm. (12.6 per cent) of crude guanine (N = 39.05 per cent).

Adenine picrate was purified by recrystallization and analyzed as follows:

0.1000 gm. substance: (Dumas) 25.3 cc. nitrogen gas at 23°C., 758 mm.
$C_5H_5N_5$. $C_6H_2(NO_2)_3OH+H_2O$. Calculated. N 29.31.
Found. " 29.10.

Guanine was analyzed as the sulfate.

0.0967 gm. of the dry substance required (Kjeldahl) 22.65 cc. 0.1 N acid.
$(C_5H_5N_5O)_2H_2SO_4$. Calculated. N 34.99.
Found. " 34.96.

Pancreas Nucleic Acid.

0.1037 gm. substance: 0.1382 gm. CO_2 and 0.0408 gm. H_2O.
0.1866 " " required (Kjeldahl) 19.15 cc. 0.1 N acid.
0.2799 " " : 0.0914 gm. $Mg_2P_2O_7$.
Found. C 36.34, H 4.40, N 14.37, P 9.10.

Bases.—The crude substance (35.0 gm.) gave 8.0 gm. (23 per cent) of crude adenine picrate and 4.0 gm. (11.5 per cent) of crude guanine.

Adenine picrate was recrystallized once and analyzed as follows:

0.1000 gm. substance: (Dumas) 26 cc. nitrogen at 26°C., 752 mm.
$C_5H_5N_5$. $C_6H_2(NO_2)_3OH+H_2O$. Calculated. N 29.31.
Found. " 29.33.

Guanine was identified as the free base.

0.0979 gm. substance required (Kjeldahl) 32.10 cc. 0.1 N acid.
$C_5H_5N_5O$. Calculated. N 46.35.
Found. " 45.90.

Liver Nucleic Acid.

0.1094 gm. substance: 0.1432 gm. CO_2 and 0.0396 gm. H_2O.
0.1800 " " required (Kjeldahl) 18.55 cc. 0.1 N acid.
0.2000 " " : 0.0924 gm. $Mg_2P_2O_7$.
Found. C 35.69, H 4.05, N 15.25, P 9.05.

Bases.—The crude acid (33 gm.) gave 9.0 gm. (30 per cent) of adenine picrate and 3.9 gm. (11.8 per cent) of crude guanine (by error was not analyzed).

Adenine picrate was recrystallized once and analyzed as follows:

0.1000 gm. substance: (Dumas) 26.4 cc. nitrogen at 29°C., 753 mm.
$C_5H_5N_5$. $C_6H_2(NO_2)_3OH+H_2O$. Calculated. N 29.31.
Found. " 29.57.

Guanine was analyzed as the free base.

0.0963 gm. substance required (Kjeldahl) 31.75 cc. 0.1 N acid.
$C_5H_5N_5O$. Calculated. N 46.35.
Found. " 46.15.

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BENZYLIDENE-ETHYL-CHITOSAMINATE AND BENZYLIDENE-ETHYL-DIAZOGLUCONATE (MANNONATE).

BY P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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Levene and La Forge[1] observed that benzylidene-ethyl-chitosaminate hydrochloride on cautious treatment with sodium nitrite, is converted into the corresponding diazo derivative. On the other hand, it has been known that chitosaminic acid and chitosamine are converted by the same reagent, not into a mixture of two epimeric deaminized derivatives, but each into a single derivative. To Levene and La Forge it seemed possible to regard the diazo derivative as an intermediate substance in the process of deamination. However, there existed no experimental proof showing the transformation of the diazo esters of the sugar acids into a single sugar acid and not into a pair of epimers.

There also existed no experimental data regarding the character of the substances which would result from the substitution of the diazo group by hydrogen chloride or bromide.

In the present work the diazo compound was hydrolyzed and the resulting hydroxy-acid identified, on the other hand, the diazo compound was converted into the bromo and chloro compounds and the chloro derivative again converted into a 2-aminohexonic acid.

On hydrolysis of the diazo derivative with dilute acetic acid the formation of only one derivative was observed; namely, benzylidene-ethyl-gluconate. This was identified in the form of saccharic acid. However, in one experiment, anhydrosaccharic acid was isolated. The bromo and chloro derivatives were prepared each with a constant melting point and constant specific rotation, showing that in each instance only one substance and not a pair of epimers had been formed. The latter conclusion is further substantiated by the fact that the

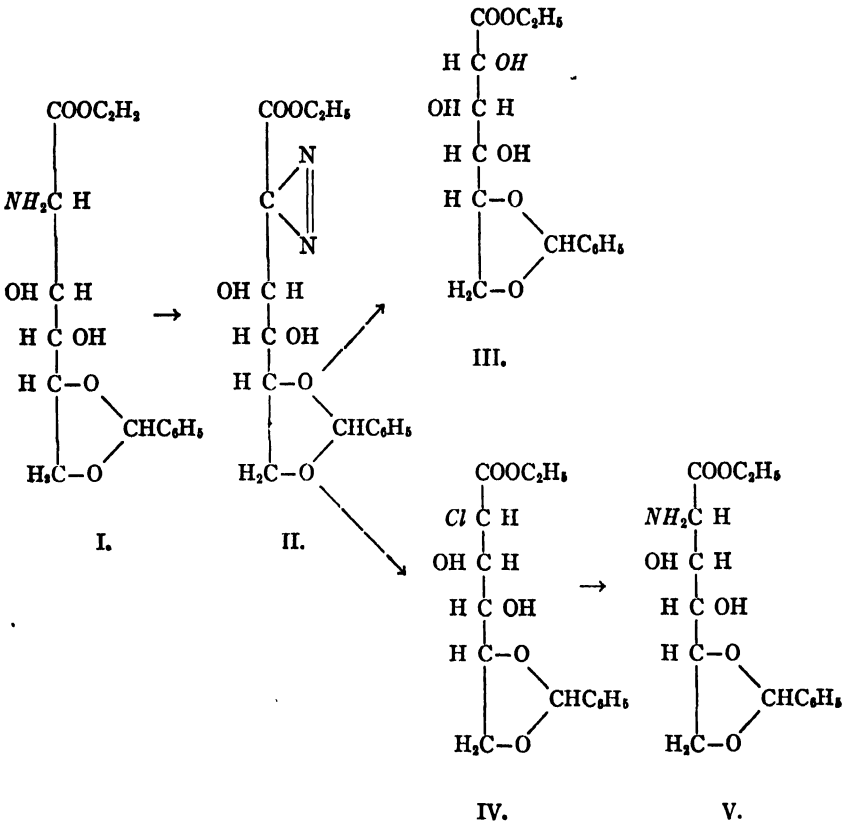
[1] Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1915, xxi, 345.

chloro derivative on treatment with ammonia gave but one chitosaminic acid (2-amino-mannonic) and not a mixture of two-amino-hexonic acids. One may be inclined to explain these reactions by the presence in the molecule of three asymmetric carbon atoms, on the other hand, this assumption is not binding since Fischer obtained two epimeric halogen derivatives, when bromine or chlorine was added to glucal.

Regarding the direction of the rotation of carbon atom 2 in the derivatives of the diazo compound, the following was observed. On hydrolysis of the compound with dilute acids a substance resulted in which the rotation of the carbon atom 2 was in the opposite direction from that of the carbon atom 2 of chitosaminic acid. On the other hand, in the bromo, chloro, and amino derivatives, the rotation of the carbon atom 2 was the same as in the original chitosaminic acid. It is here accepted that in the chloro and bromo derivative the direction of the rotation of the carbon atom 2 determines the direction of rotation of the acid. Hence for the present for the chloro and bromo derivatives, the configuration of mannonic acid is assumed. On this assumption chitosaminic acid passes through the diazo derivative into the chloro derivative and back into the amino-acid apparently without Walden inversion. On the other hand, deamination through only the diazo derivative undergoes the Walden inversion in the same way as on direct deamination.

In connection with the Walden inversion it is interesting to note that it occurs in this instance both in the acid and in its ester. In the majority of amino-acids according to the observation of Fischer, the inversion occurs only in the acid and not in the ester. In the amino-acids where acid and ester both gave rise to the same hydroxy-acid, Fischer's original view was that no inversion occurred in either, and later he reversed his opinion accepting an inversion in both. In the present instance chitosaminic acid and ester give a hydroxy-acid in which the carbon atom 2 rotates to the right, whereas chitosamine leads to a hydroxy-acid in which the carbon atom 2 rotates to the left. On the basis of considerations discussed in another article it was assumed that the inversion occurred in chitosaminic acid, hence it also occurs in the ester. This fact is a corroboration of the later theory of Fischer.

The set of reactions here described are represented by the following graphic expression:

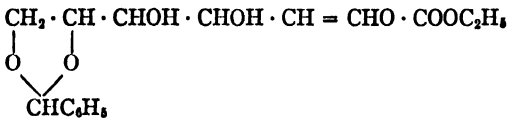


In the course of the work some derivatives were prepared which have no direct bearing on the present problem, but eventually they may become of theoretical value. These compounds are: Benzylidene-chitosaminic acid, benzylidene-ethyl-chitosaminate (this was reconverted into its hydrochloride), benzylidene-acetone-ethyl-chitosaminate, benzylidene-1-ethyl-2,3-anhydrogluconate (mannonate), and benzylidene-ethyl-desoxygluconate (mannonate). The last may be a mixture of gluconate and mannonate. Benzylidene-1-amino-2,3-dehydrogluconate (mannonate) and benzylidene-1-amino-2-chloromannonate were also prepared.

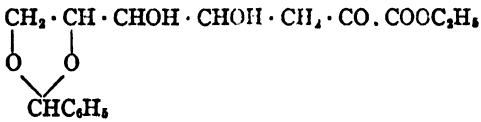
The first three substances were obtained in the process of preparation of free benzylidene-ethyl-chitosaminate. In one phase of the work it seemed as if the halogen derivative of the diazo compound was readily converted into the free amino derivative, and hence it was desirable to compare the substance obtained from the diazo compound with that from benzylidene-ethyl-chitosaminate hydrochloride.

The benzylidene-acetone-ethyl-chitosaminate was obtained accidentally, when it was attempted to recrystallize benzylidene-ethyl-chitosaminate from acetone. The condensation took place in the short time required to bring the original substance in solution. It is remarkable that the acetone is cleaved off as readily as it is condensed with the benzylidene-ethyl-chitosaminate. Benzylidene-chitosaminic acid was obtained as a by-product in the process of preparation of its ester.

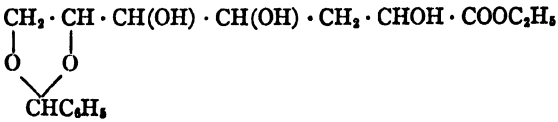
Benzylidene-1-ethyl-2,3-anhydrogluconate (mannonate) is obtained almost instantly when an alcoholic solution of benzylidene-ethyl-chitosaminate is poured into aqueous ammonia and the solution cooled to 0°C. This derivative apparently retains the enolic structure



and not the structure



since with phenylhydrazine it did not form a hydrazone, and furthermore in the presence of palladium it was readily hydrogenated giving the following substance:



(The position of the desoxy carbon atom may be either 2 or 3.)

It is not known whether this is a single substance or a mixture of two epimers. Thus attempts to convert the bromo derivatives into benzylidene-chitosaminic ester failed. The attempt to convert the free bromomannonic acid into chitosaminic acid was also not successful.

Benzylidene-1-amino-2-chloromannonate is formed when an alcoholic solution of the chloro ester is poured into an excess of aqueous ammonia and the solution is allowed to crystallize. The same substance is formed when the chloro ester is dissolved in alcohol containing ammonia gas. On the contrary, if an alcoholic solution of the chloro ester added to aqueous ammonia is heated at 95°C. for 10 hours in a sealed tube the product is benzylidene-ethyl-chitosaminate.

Since in the course of the reaction some decomposition takes place it is more conveniently identified as chitosaminic acid.

EXPERIMENTAL.

Benzylidene-Ethyl-Chitosaminate Hydrochloride.—The substance was prepared under conditions previously described. For purification it was dissolved in methyl alcohol. To the solution dry ether was added until crystallization was complete. The substance melted at 200°C. (uncorrected) and analyzed as follows:

0.1972 gm. substance: (Kjeldahl) 5.65 cc. 0.1 N acid.
0.1972 " " : (Volhard) 5.6 " 0.1 N silver nitrate.
0.020 " " : (*a*) (Van Slyke) in 4 min. 0.38 cc. nitrogen at 22°C., 753.4 mm.
0.020 gm. substance: (*b*) (Van Slyke) in 30 min. 0.59 cc. nitrogen at 22°C., 753.4 mm.

$C_{15}H_{21}NO_7HCl$.	Calculated.	N 4.04,	Amino N 4.04, Cl 10.94.
	Found.	" 4.01,	" " (*a*) 1.06, " 10.07.
			" " (*b*) 1.65.

The substance had the following rotation:

$$[\alpha]_D^{25} = \frac{-0.30° \times 100}{1 \times 1} = -30°$$

Benzylidene-Ethyl-Chitosaminate and Benzylidene-Chitosaminic Acid.—The hydrochloride (40.0 gm.) was dissolved in water (150 cc.) and an excess of an aqueous 1.0 N sodium hydroxide solution (140 cc.)

was added. On scratching the walls of the container the solution nearly solidifies into a mass consisting of curved needles. The precipitate was filtered on a suction funnel and washed a few times with water. The precipitate was dried under diminished pressure over sulfuric acid until practically dry. The yield of the dry product was about 20.0 gm. The substance consisted principally of the free benzylidene-chitosaminic ester with a small admixture of benzylidene-chitosaminic acid. The filtrate contained a larger quantity of the latter substance. This crystallized out on standing in the form of large crystalline plates. The separation of the benzylidene ester from the acid was accomplished in the following way: The dried substance (1 part) was taken up in boiling 98.5 per cent alcohol (4 parts) and filtered. The insoluble part consisted of the benzylidene-chitosaminic acid. The filtrate contained the ester. On standing it crystallized out in long prisms. For analysis it was recrystallized twice from small volumes of 98.5 per cent alcohol. It melted at 120°C. (corrected) and analyzed as follows:

0.0770 gm. substance: 0.1638 gm. CO_2 and 0.0432 gm. H_2O.
0.1288 " " required (Kjeldahl) 4.45 cc. 0.1 N acid.

$C_{15}H_{20}NO_6$. Calculated. C 57.85, H 6.80, N 4.50.
Found. " 57.94, " 6.28, " 4.83.

The substance in methyl alcoholic solution had the following rotation:

$$[\alpha]_D^{20} = \frac{-0.59° \times 100}{1 \times 1} = -59°$$

The benzylidene-chitosaminic acid was purified by dissolving in hot water and adding 98.5 per cent alcohol to slight opalescence. On standing the substance crystallized in large prismatic plates. After two or three crystallizations the substance analyzed correctly. It melted at 230°C. (uncorrected) and analyzed as follows:

0.1084 gm. substance: 0.2008 gm. CO_2 and 0.0594 gm. H_2O.
0.1859 " " required (Kjeldahl) 6.55 cc. 0.1 N acid.
0.0186 " " : (Van Slyke) 1.75 cc. nitrogen at 24.0°C., 765 mm.

$C_{13}H_{17}NO_6$. Calculated. C 55.09, H 6.05, N 4.94, Amino N 4.94.
Found. " 55.40, " 6.19, " 4.93, " " 4.92.

The substance had in aqueous solution the following optical rotation:

$$[\alpha]_D^{20} = \frac{-0.28^\circ \times 100}{1 \times 1} = -28^\circ$$

Benzylidene-Acetone-Ethyl-Chitosaminate.—This substance was obtained, incidentally, in an experiment aiming to separate benzylidene-chitosaminic acid from its ester. The crude material (40.0 gm.) was suspended in dry acetone and digested on a boiling water bath. A part (3.0 gm.) remained undissolved. This substance proved to be the benzylidene-chitosaminic acid. The mother liquor was allowed to stand in a vacuum desiccator over sulfuric acid. Soon very large prismatic crystals began to form on the edge of the liquid and after a few days crystallization seemed complete. The yield was about 20 gm. From the mother liquor on standing, further crystallization took place. For purification the material may be recrystallized either from absolute alcohol or from acetone. The substance melts sharply at 138°C. It analyzed as follows:

0.1134 gm. substance: 0.2380 gm. CO_2 and 0.732 gm. H_2O.
0.1986 " " required (Kjeldahl) 5.55 cc. 0.1 N acid.
0.020 " " : (Van Slyke) after 30 min., 0.87 cc. nitrogen at 24°C., 760 mm.

$C_{15}H_{22}NO_6$. Calculated. C 61.50, H 7.18, N 3.98.
Found. " 61.37, " 7.22, " 3.91.

The optical rotation of the substance was:

$$[\alpha]_D^{20} = \frac{-0.70^\circ \times 100}{1 \times 1} = -70^\circ$$

The substance is readily reconverted into the hydrochloride of benzylidene-ethyl-chitosaminate. It (2.0 gm.) was dissolved in 20 cc. of dry methyl alcohol; to this solution 20 cc. of 0.6 N hydrochloric acid in dry ether were added, then dry ether until slight opalescence. On standing a substance crystallized which melted at 202°C. (uncorrected) and analyzed as follows:

0.1077 gm. substance: 0.2044 gm. CO_2 and 0.0626 gm. H_2O.
0.1982 " " required (Kjeldahl) 5.55 cc. 0.1 N acid.

$C_{15}H_{22}NO_6HCl$. Calculated. C 51.72, H 6.34, N 4.04.
Found. " 51.75, " 6.50, " 3.99.

Diazo Derivative of Benzylidene-Ethyl-Chitonate.—The original method for the preparation of the diazo ester was slightly modified. The hydrochloride of benzylidene-ethyl-chitosaminate (20 gm.) is dissolved in 250 cc. of water and chilled to the same temperature. The two solutions are combined and to the resulting solution glacial acetic acid (30.0 cc.) is added. The contents of the flask are practically solidified. The product at this phase seems colorless. When filtered off, however, it was a light yellow. The product is dissolved in ether and the ethereal solution is washed (four times) in a separatory funnel with a cold solution of sodium carbonate and then (three times) with water. The ethereal solution is dried by means of anhydrous sodium sulfate and concentrated under diminished pressure to a volume of about 35 cc., then transferred into an evaporating dish which is placed in a vacuum desiccator over sulfuric acid. The solution is evaporated to dryness leaving a light yellow mass which is readily pulverized. The powder is exhaustively extracted with low boiling petroleum ether and is then analytically pure.

The nitrogen estimation is easily carried out by the process described by Levene and Mikeska.[2]

Several samples were analyzed in this manner with the following results:

Sample 1. 0.020 gm. substance: 1.60 cc. nitrogen at 26°C., 760 mm.
" 2. 0.020 " " : 1.57 " " " 26° " 760 "
" 3. 0.020 " " : 1.54 " " " 26° " 760 "

$C_{15}H_{18}O_6N_2$. Calculated. N 8.69.
Found, Sample 1. " 8.88.
" " 2. " 8.71.
" " 3. " 8.55.

The optical rotation of the substance was the following:

$$[\alpha]_D^{25} = \frac{-1.00° \times 100}{2 \times 1} = -50°$$

Hydrolysis of the Diazo Derivative.—The product resulting from hydrolysis of the diazo derivative depends on the conditions of the reaction. If the reaction takes place in the absence of water or in

[2] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1922, lii, 485.

organic solvents containing only a small proportion of water the product is apparently ethyl-2,3-anhydrogluconate, otherwise ethyl-gluconate or ethyl-1,4-anhydrogluconate is formed as the principal product. It was found difficult to isolate and identify the original reaction product, hence this was further oxidized by means of nitric acid. In the conditions in which only the unsaturated acid formed, the product of oxidation was mesotartaric acid, under other conditions the product of oxidation was either saccharic or anhydrosaccharic acid. The conditions of reaction were as follows:

The diazo compound (20 gm.) was suspended in 400 cc. of distilled water and glacial acetic acid (20.0 gm.) was added. The flask is placed near a hot water bath and from time to time warmed on the water bath so as to maintain a continuous but gentle evolution of nitrogen gas. The reaction was considered completed when a dark yellow oil (benzaldehyde) settled out on the bottom of the flask. The oil is separated in a separatory funnel, the aqueous portion is extracted with ether and concentrated to nearly dryness under diminished pressure. The residue is dissolved in water and again concentrated under diminished pressure. The operation is repeated four times. In this manner the greatest part of the acetic acid is removed. The final residue is taken up in 40.0 cc. of water, an equal volume of concentrated nitric acid is added, and the solution is allowed to stand over night. It is then transferred to a clock-glass and evaporated to dryness on a water bath. The residue is dissolved in nitric acid, diluted with an equal volume of water, and the solution evaporated to dryness. The residue is then dissolved in water and the process repeated. The final product is dissolved in water, again shaken out with ether, and finally converted into the calcium salt.

The yield of the calcium salt is about 5 to 6 gm. from 100 gm. of the diazo compound. In the earlier experiments the calcium salt was purified by repeatedly dissolving it in water containing the requisite amount of oxalic acid and reconverting it into the calcium salt until a product with a maximum optical rotation of about $[\alpha]_D^{20} = + 24.0°$ was obtained. Under these conditions anhydrosaccharic acid was obtained.

When the calcium salt was only once recrystallized and then converted into the acid potassium salt, the salt of saccharic acid was obtained.

The analytical results obtained on anhydrosaccharic acid were as follows:

0.1000 gm. substance: 0.0348 gm. K_2SO_4.

$C_6H_7O_7K+H_2O$.	Calculated.	K 15.70.
	Found.	" 15.61.

The optical rotation of the substance was:

$$[\alpha]_D^{20} = \frac{+0.63^\circ \times 100}{1 \times 1} = +63^\circ$$

The analytical results obtained on the salt of the saccharic acid were as follows:
0.1000 gm. substance: 0.0352 gm. K_2SO_4.

$C_6H_9O_8K$.	Calculated.	K 15.70.
	Found.	" 15.80.

The optical rotation of the substance is as follows:

$$[\alpha]_D^{20} = \frac{+0.07^\circ \times 100}{1 \times 1} = +7^\circ$$

Benzylidene-1-Ethyl-2-Bromomannonate.—The diazo compound (20.0 gm.) is suspended in 400.0 cc. of anhydrous (over sodium) ether, and dry ether saturated with hydrogen bromide gas is added as long as the diazo compound is dissolved and the yellow color has disappeared. The ethereal solution is then washed with a saturated solution of sodium carbonate cooled to 0°C. The operation is repeated until all hydrobromic acid is removed from the ethereal solution. The ethereal solution is then dried with anhydrous sodium sulphate, concentrated to a small volume, and the bromo compound is precipitated with ligroin (80–90°C.). The crude compound is obtained analytically pure after several recrystallizations, from a mixture of ether and ligroin. The yield was between 25 to 40 gm. from 100.0 gm. of the diazo compound. The substance melted at 119°C. (corrected) and analyzed as follows:

0.1040 gm. substance: 0.1820 gm. CO_2 and 0.0480 gm. H_2O.
0.2000 " " : 0.1016 gm. AgBr.

$C_{18}H_{18}BrO_6$.	Calculated.	C 47.99, H 5.10, B 21.30.
	Found.	" 47.25, " 5.16, " 21.56.

The optical rotation of the substance was:

$$[\alpha]_D^{25} = \frac{-0.66^\circ \times 100}{1 \times 2} = -33^\circ$$

Benzylidene-1-Ethyl-2,3-Anhydromannonate.—The substance was obtained in an experiment which aimed to convert the bromo compound into the corresponding amino derivative.

The bromo compound (30.0 gm.) is dissolved in 35 cc. of 98.5 per cent alcohol and poured into 30.0 cc. of concentrated aqueous ammonia. After standing for 5 to 10 minutes at room temperature the substance solidifies on cooling into a solid mass consisting of long microscopic needles. The material is filtered, dried in a vacuum desiccator over sulfuric acid and then recrystallized from 35 per cent alcohol. This operation is repeated several times. Finally the product is twice recrystallized in its own weight of boiling 98.5 per cent alcohol. The final product melted at 122.5°C. (corrected) and analyzed as follows:

0.1130 gm. substance: 0.2522 gm. CO_2 and 0.0594 gm. H_2O.

$C_{15}H_{18}O_6$. Calculated. C 61.21, H 6.12.
Found. " 60.86, " 5.88.

The optical rotation of the substance was:

$$[\alpha]_D^{25} = \frac{-1.10^\circ \times 100}{1 \times 1.5} = -73.3^\circ$$

Benzylidene-Ethyl-Desoxygluconate (Mannonate).—The previous substance (3.0 gm.) was dissolved in 10 cc. of 98.5 per cent alcohol and saturated with hydrogen gas in the presence of Paal's colloidal palladium. The substance absorbed the theoretical volume of hydrogen. The operation was completed in 72 hours. The product was filtered. The greatest part of the palladium, however, remained in colloidal solution. Hence the filtrate was concentrated to dryness under diminished pressure. The residue was dissolved in a little boiling alcohol with charcoal, filtered, and allowed to evaporate to dryness. This operation was repeated three times when a perfectly colorless product was obtained. It melted at 126°C. (corrected) and analyzed as follows:

0.1081 gm. substance: 0.2274 gm. CO_2 and 0.0642 gm. H_2O.
$C_{15}H_{20}O_6$. Calculated. C 60.81, H 6.76.
Found. " 60.91, " 7.05.

The optical rotation of the substance was:

$$[\alpha]_D^{20} = \frac{-0.52^\circ \times 100}{1 \times 2} = -26^\circ .$$

Benzylidene-1-Amino-2,3-Anhydrogluconate (Mannonate).—When a solution of the bromo compound in alcohol is added to ammonia water following exactly the same conditions as for the preparation of the benzylidene-ethyl-anhydrogluconate, and is allowed to stand at 0°C., the solution remains either liquid or partly gelatinous. On further cooling in an ice-alcohol mixture the contents of the flask turn into a gelatinous mass. This is best filtered and washed with water; it then acquires a white granular character. After it is dried in a vacuum desiccator (over soda-lime) to complete dryness it can be recrystallized from absolute alcohol. The process may be repeated until the product is analytically pure. The substance melted at 230°C. and analyzed as follows:

0.1106 gm. substance: 0.2380 gm. CO_2 and 0.0586 gm. H_2O.
0.0993 " " required (Kjeldahl) 3.64 cc. 0.1 N acid.
$C_{13}H_{15}NO_5$. Calculated. C 58.84, H 5.70, N 5.28.
Found. " 58.68, " 5.93, " 5.14.

The substance had the following rotation:

$$[\alpha]_D^{20} = \frac{+1.30^\circ \times 100}{1 \times 2} = +65^\circ$$

Action of Aqueous Ammonia on Benzylidene-2-Bromo-Ethyl-Gluconate.—Benzylidene-2-bromo-ethyl-gluconate (5.0 gm.) was suspended in 100 cc. of 2 per cent sulfuric acid and placed on a water bath for 1 hour. The benzaldehyde settled out on the bottom of the flask in the form of an oil. The oil was extracted by means of ether and the aqueous solution freed from sulfuric acid quantitatively by means of barium hydroxide. The aqueous solution was then concentrated to a volume of 20 cc., an equal volume of concentrated ammonia water was added, and the solution was kept in a sealed tube at 100°C. for 24 hours. The resulting solution after the removal of

the ammonia, showed the presence only of a few mm. of amino nitrogen.

Benzylidene-1-Ethyl-2-Chlorogluconate.—The substance was prepared in the same manner as the corresponding bromo compound, with the exception that ether saturated with hydrogen chloride gas was used. The yield was 10.0 gm. from 100.0 gm. of the diazo compound. It melted at 127°C. and analyzed as follows:

0.1062 gm. substance: 0.2122 gm. CO_2 and 0.0584 gm. H_2O.
0.2024 " " : 0.0858 " AgCl.
$C_{15}H_{19}ClO_6$. Calculated. C 54.46, H 5.8, Cl 10.74.
Found. " 54.69, " 6.11, " 10.48.

The rotation of the substance was:

$$[\alpha]_D^{20} = \frac{-0.20° \times 100}{1 \times 1} = -20°$$

Benzylidene-1-Amino-2-Chloromannonate.—The substance described in the previous section (5.0 gm.) was dissolved in 5 cc. of 98.5 per cent alcohol and transferred into 4.0 cc. of aqueous ammonia. The solution was allowed to stand over night and then concentrated in a vacuum desiccator. A deposit, consisting of microscopic needles, was formed. This was recrystallized first from 35 per cent alcohol and finally from 98.5 per cent alcohol until the substance gave a negative test with Nessler's reagent. The substances melted at 197°C. (corrected) and analyzed as follows:

0.1022 gm. substance: 0.1938 gm. CO_2 and 0.0540 gm. H_2O.
0.1857 " " : 0.0844 " AgCl.
0.1887 " " required (Kjeldahl) 5.95 cc. 0.1 N acid.
$C_{13}H_{16}NO_6Cl$. Calculated. C 51.73, H 5.35, N 4.69, Cl 11.75.
Found. " 51.72, " 5.52, " 4.41, " 11.23.

The substance had the following rotation:

$$[\alpha]_D^{20} = \frac{-0.23° \times 100}{1 \times 1} = -23°$$

Conversion of the Benzylidene-1-Ethyl-2-Chloromannonate into Chitosaminic Acid.—The chloro compound (3.0 gm.) was dissolved in 4 cc. of 98.5 per cent alcohol and the solution added to 4.0 cc. of aqueous ammonia. This is sealed in a tube and heated at 95°C. for 10 hours.

At the end of that time the tube is allowed to cool and the solution which has then turned dark brown is concentrated to nearly dryness under diminished pressure. The residue is taken up in 2 per cent sulfuric acid and boiled over a flame for 15 minutes. It is then allowed to cool and washed with ether, (in a separatory funnel) to remove the benzaldehyde. The resulting aqueous solution is freed from hydrochloric acid and ammonia in the usual way and concentrated to nearly dryness. The residue is dissolved in a little water, acetone is added to the solution until an oil settles out, and all is warmed on a water bath until crystallization begins. Prior to treatment with acetone a small sample of the aqueous solution was used for an amino nitrogen determination according to Van Slyke. On the basis of this estimation the solution contained 0.9 gm. of chitosaminic acid. However, from five experiments only 2.5 gm. of chitosaminic acid crystallized. After one recrystallization the substance was analytically pure. It analyzed as follows:

0.1108 gm. substance: 0.1508 gm. CO_2 and 0.0674 gm. H_2O.
0.0670 " " required (Kjeldahl) 3.45 cc. 0.1 N acid.
$C_6H_{13}NO_6$. Calculated. C 36.92, H 6.66, N 7.18.
Found. " 37.11, " 6.80, " 7.21.

The rotation of the substance was:

$$[\alpha]_D^{25} = \frac{-0.15^\circ \times 100}{1 \times 1} = -15^\circ$$

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UNSATURATED FATTY ACIDS OF BRAIN CEPHALINS.

BY P. A. LEVENE AND IDA P. ROLF.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 10, 1922.)

Cephalin was recognized as a substance distinct from lecithin by Thudichum.[1] According to this author, the differences in the properties of the two substances were due to the differences in the structure of the fatty acids and of the bases which enter into their respective molecules. As regards the structure of the base, the views of Thudichum were confirmed by the more recent and more modern work. His views on the nature of the fatty acids also, seemed to have been corroborated by later work of Cousin,[2] Falk,[3] and especially that of Parnas.[4]

Recent work on the fatty acids of lecithin has led to the discovery that at least two and probably three unsaturated and two saturated acids are present in the mixed lecithins.[5,6,7] In the light of this experience it seemed desirable to subject the problem of the nature of the fatty acids of cephalin to a new investigation.

The material used for the present work was entirely free from lecithin and neutral fats. It was also entirely free from substances containing non-amino nitrogen. As is seen from its elementary composition this material consisted in part of cephalin which had undergone partial hydrolysis. It was as pure as the best that had been used by the previous workers. From this material pure cephalin undoubtedly can be prepared. The purification is time-consuming

[1] Thudichum, J. L. W., A treatise on the chemical constitution of the brain, London, 1884.

[2] Cousin, M. H., *Compt. rend. Soc. biol.*, 1906, lxi, 23.

[3] Falk, F., *Biochem. Z.*, 1909, xvi, 187.

[4] Parnas, J., *Biochem. Z.*, 1909, xxii, 411; 1913, lvi, 17.

[5] Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1922, li, 285.

[6] Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1922, li, 507.

[7] Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1921, xlvi, 363.

and results in much loss of material. In the course of the work the highly unsaturated acids may undergo oxidation. Owing to these considerations the purification of the material was limited to the stage in which it was free from non-amino nitrogen, in other words, free from lecithin. Such material may yield fewer, not a greater number of fatty acids than are present in the pure cephalin.

Following the course of analysis as employed in the work on lecithin, it was possible to isolate from cephalin two unsaturated fatty acids; namely, oleic and arachidonic acid. The oleic acid was separated first as a barium salt (mixed with the saturated acids). This was then converted into the free acid and as such it was identified by the iodine number and by the fact that on hydrogenation it yielded stearic acid. The presence of arachidonic acid was demonstrated by the isolation of its octabromide, and by the isolation of arachidic acid from the product of hydrogenation of the unsaturated fatty acids.

Since a minor fraction only, of the highly unsaturated acids was converted into the insoluble octabromides, there still remains the possibility of the discovery of other unsaturated acids. At present no evidence is available in support of the assumption that linolic acid is the principal unsaturated acid of cephalin.

From the saturated fatty acid fraction, stearic acid was isolated in pure condition. This fraction, however, also contained other acids of a different composition. This material is now under further investigation.

EXPERIMENTAL.

A. Preparation of Cephalin.

Ox brains were extracted first with acetone and subsequently with ether containing 5 per cent of water. From the concentrated ethereal extract, the bulk of the cerebrosides was removed by filtration at 0°C. The filtrate was then fractionated with alcohol. The precipitate after purification yielded material which contained all of its nitrogen as amino nitrogen. Two methods of purification were employed on each sample. The first is that described by Levene and Ingvaldson.[8] The crude cephalin is dissolved in glacial acetic acid. This solution is

[8] Levene, P. A., and Ingvaldsen, T., *J. Biol. Chem.*, 1920, xliii, 359.

allowed to stand for 18 hours at 15°C. The precipitate, consisting of cholesterol and cerebrosides, is removed by filtration. To the filtrate about 10 volumes of alcohol are added and again the mixture is allowed to stand over night at 0°C. and the resultant precipitate removed. The filtrate, after concentration *in vacuo*, is emulsified with water and precipitated with acetone. This method of purification yields material with a markedly higher carbon content than that of the original mixture.

The second method of purification permits the separation of two types of material, one, the amino content of which is greatly increased, and one of a greater carbon content than the original material. Crude cephalin is dissolved in a minimal quantity of warm ether. To this, warm alcohol is gradually added, not, however, in sufficient quantity to cause precipitation. A saturated alcoholic solution of cadmium chloride is added, until no further precipitation occurs. The mother liquor (A) is decanted from the precipitate (B) and concentrated to dryness under diminished pressure. The residue is extracted with ether and the ethereal solution, after filtration from the inorganic precipitate, is again concentrated, emulsified with water, and precipitated with acetone. The yields of such precipitates have been as high as 20 per cent of the starting material. Since this consists only of the more highly unsaturated fraction, great variation is experienced between different lots of cephalin. The precipitate (B) referred to above, contains lecithin cadmium chloride (C) and "cephalin" (D) of a lower carbon content. The latter is separated by its solubility in glacial acetic acid. This procedure constitutes the simplest means thus far used for the preparation of "cephalin" of high amino content.

Of such fractionation the following results are typical:

No. 216 was the alcohol-soluble fraction of an ethereal extract. It was purified by the glacial acetic acid-alcohol method, and analyzed as follows:

0.1060 gm. substance: 0.0926 gm. H_2O, 0.2426 gm. CO_2, and 0.0126 gm. ash.
0.1880 " " required (Kjeldahl) 2.85 cc. 0.1 N acid.
0.2819 " " : (fusion) 0.0412 gm. $Mg_2P_2O_7$.
0.2 gm. was dissolved in glacial acetic acid.

5 cc. of this solution required (Kjeldahl) 1.25 cc. 0.1 N acid.
2 " " " " : (Van Slyke) 0.71 cc. N_2 at 24°C., 766.5 mm.
0.2781 gm. substance absorbed 0.2154 gm. iodine.
Found (No. 216). C 64.10, H 9.92, N 2.02, P 4.07.

$$\frac{\text{Amino } N_2}{\text{Total } N_2} = \frac{56}{100} \quad \text{Iodine No.} = 80$$

No. 216 was fractionated through the use of cadmium chloride, as described above. The material obtained from the mother liquor (A) analyzed as follows:

No. 220. 0.1090 gm. substance: 0.0984 gm. H_2O, 0.2574 gm. CO_2, and 0.0112 gm. ash.
0.1838 gm. substance required (Kjeldahl) 2.65 cc. 0.1 N acid.
0.2757 " " : (fusion) 0.0400 gm. $Mg_2P_2O_7$.
0.2 gm. substance was dissolved in glacial acetic acid.
5 cc. of this solution required (Kjeldahl) 0.75 cc. 0.1 N acid.
2 " " " " : (Van Slyke) 0.55 cc. N_2 at 24°C., 764.5 mm.
0.2724 gm. substance absorbed 0.2630 gm. iodine.
Found (No. 220). C 65.05, H 10.23, N 2.01, P 4.04.

$$\frac{\text{Amino } N_2}{\text{Total } N_2} = \frac{73}{100} \quad \text{Iodine No.} = 96$$

The lecithin cadmium chloride salt (C), insoluble in glacial acetic acid, contained 25 per cent amino nitrogen and had an iodine number of 40.

That fraction (D) which was extracted by the glacial acetic acid had the following composition:

0.1040 gm. substance: 0.0884 gm. H_2O, 0.2306 gm. CO_2, and 0.0144 gm. ash.
0.1867 " " required (Kjeldahl) 2.70 cc. 0.1 N acid.
0.2801 " " : (fusion) 0.0410 gm. $Mg_2P_2O_7$.
0.2 gm. substance was dissolved in glacial acetic acid.
5 cc. of this solution required (Kjeldahl) 0.75 cc. 0.1 N acid.
2 " " " " : (Van Slyke) 1.10 cc. N_2 at 22°C., 773.2 mm.
0.2915 gm. substance absorbed 0.2249 gm. iodine.
Found (No. 218). C 62.11, H 9.90, N 2.02, P 4.07.

$$\frac{\text{Amino } N_2}{\text{Total } N_2} = \frac{103}{100} \quad \text{Iodine No.} = 77$$

This material served as the source of the fatty acids to be described later. The cephalin was boiled for 12 hours with 10 per cent hydro-

chloric acid, and the fatty acids were extracted with ether. Owing to the presence of considerable resinous material which was not wholly removed even by this method, it was found expedient to esterify the mixed acids and extract the esters with ether. This solution was thoroughly washed with a solution of dilute sodium carbonate, and repeatedly filtered. The esters were saponified with barium hydroxide and the barium salts separated into two fractions by the method described in a previous publication.[6]

B. Saturated Acids.

From the barium salts which were insoluble in benzene-alcohol, the acids were liberated. These acids, consisting of mixed saturated and oleic acids, were converted into lead salts and the lead oleate was extracted by a large volume of ether. The residual salts of the saturated acids were dissolved in toluene and from them the free acids were liberated by hydrogen sulfide. They were then esterified and the methyl esters after recrystallization were fractionated by distillation at a pressure of 1.9 mm.

The esters were saponified, and analyses, melting points, and molecular weight determinations made on the free acids. The drying, analyses, and molecular weight determinations were carried out in the manner described in previous papers.[6,7] The melting points, however, were taken in a bath which was continuously stirred. A corrected thermometer was used, and the time interval per degree rise in temperature was 6 seconds.

No. 572. 0.1006 gm. substance: 0.1166 gm. H_2O and 0.2788 gm. CO_2.
1.0056 " " required for neutralization 7.05 cc. 0.5 N NaOH, corresponding to a mol. wt. of 285.

No. 562. 0.0993 gm. substance: 0.1186 gm. H_2O and 0.2766 gm. CO_2.
1.1524 " " required for neutralization 7.96 cc. 0.5 N NaOH.

No. 563. 0.1010 gm. substance: 0.1162 gm. H_2O and 0.2724 gm. CO_2.
1.0378 " " required for neutralization 7.10 cc. 0.5 N NaOH.

No. 564. The residue was insufficient for either molecular weight or analysis.

$C_{18}H_{36}O_2$. Calculated. C 75.98, H 12.76 (Mol. wt. = 284. Melting point = 70–71°C.)

	No.	Boiling point of ester. Pressure 1.9 mm.	Weight of ester.	Analysis of acid.		Molecular weight of acid.	Melting point of acid.
				C	H		
		°C.	gm.				°C.
Found.	572	165–172	5	75.57	12.97	285	70–71
"	562	172	12	75.96	13.36	289	64–65
"	563	175–185	5	74.03	12.87	292	66–67
"	564	Residue.	1				76

C. Oleic Acid.

The lead salt which was extracted by ether from the saturated lead salts, was converted into the free acid. This was a light yellow mobile liquid. 0.2540 gm. of substance absorbed 0.2273 gm. of iodine, corresponding to an iodine number of 90. The iodine number of oleic acid is 90.

This substance was hydrogenated and the saturated acid had the following analysis, molecular weight, and melting point:

0.1008 gm. substance: 0.1170 gm. H_2O and 0.2800 gm. CO_2.
0.9492 " " required for neutralization 6.67 cc. 0.5 N NaOH.
It melted at 70–71°C.
$C_{18}H_{36}O_2$. Calculated. C 75.98, H 12.76. (Mol. wt. = 284. Melting point = 70–71°C.)
Found (No. 571). C 75.75, H 12.99. (Mol. wt. = 284. Melting point = 70–71°C.)

D. The More Unsaturated Acids.

The barium salts which were soluble in the benzene-alcohol mixture were converted into free acids. As such they formed a dark mobile liquid having an iodine number of 140.

0.2611 gm. of substance absorbed 0.3677 gm. of iodine. 60 gm. of No. 517 were esterified and the ethyl esters hydrogenated by Paal's method. The unsaturated esters were recrystallized from alcohol, and fractionated by distillation at 1.9 mm. pressure. After saponification, the acids gave the analyses, molecular weights, and melting points recorded below.

No. 549. (Mixed esters before fractionation after saponification to the acid.) 0.1008 gm. substance: 0.1062 gm. H_2O and 0.2642 gm. CO_2.
No. 550. 0.0978 gm. substance: 0.0772 gm. H_2O and 0.2080 gm. CO_2.
0.9637 " " required for neutralization 4.70 cc. 0.5 N NaOH

No. 551. 0.1010 gm. substance: 0.1154 gm. H_2O and 0.2562 gm. CO_2.
1.0130 " " required for neutralization 5.85 cc. 0.5 N NaOH.

No. 552. 0.1008 gm. substance: 0.1156 gm. H_2O and 0.2828 gm. CO_2.
1.0721 " " required for neutralization 5.73 cc. 0.5 N NaOH.

No. 553. 0.1002 gm. substance: 0.1146 gm. H_2O and 0.2830 gm. CO_2.
0.9228 " " required for neutralization 6.12 cc. 0.5 N NaOH.

No. 554. 0.1009 gm. substance: 0.1190 gm. H_2O and 0.2838 gm. CO_2.
1.0647 " " required for neutralization 6.25 cc. 0.5 N NaOH.

No. 555. 0.1010 gm. substance: 0.1204 gm. H_2O and 0.2854 gm. CO_2.
1.0727 " " required for neutralization 6.00 cc. 0.5 N NaOH.

No. 556. 0.1008 gm. substance: 0.1214 gm. H_2O and 0.2862 gm. CO_2.
1.0645 " " required for neutralization 5.95 cc. 0.5 N NaOH.

Nos. 555 and 556 were combined and purified by conversion into the lead salt. The liberated acids were fractionally distilled and the lower fraction (No. 573) gave the following analysis and neutralization value.

0.0865 gm. substance: 0.1024 gm. H_2O and 0.2432 gm. CO_2.
0.3626 " " required for neutralization 2.30 cc. of 0.5 N NaOH.
$C_{18}H_{36}O_2$. Calculated. C 75.98, H 12.76. (Mol. wt. = 284. Melting point = 70–71°C.)
$C_{20}H_{40}O_2$. Calculated. C 76.95, H 12.81. (Mol. wt. = 312. Melting point = 75–77°C.)

	No.	Boiling point of ester. Pressure 1.9 mm.	Weight of ester.	Analysis of acid.		Molecular weight of acid.	Melting point of acid.
				C	H		
		°C.	gm.				°C.
Found.	549 (Mixed esters.)			71.47	11.79		
"	550	170–185	3	57.99	8.83	410	
"	551	185–190	8	69.17	12.78	347	62–6
"	552	185–195	6	76.51	12.83	373	58–5
"	553	190–207	8	77.04	12.80	316	62–6
"	554	195–205	5	76.70	13.19	341	66–6
"	555	210–220	4	77.05	13.33	357	72
"	556	220–230	2.5	77.42	13.47	374	72
"	573			76.67	13.29	315	76

E. Bromine Addition Products of the More Highly Unsaturated Acids.

50 gm. of No. 217 were brominated by the method described in a previous paper.[6] The precipitated bromide, after repeated extraction with ether, weighed 15 gm., and gave the following analysis:

No. 521. 0.1062 gm. substance: 0.0348 gm. H_2O and 0.0984 gm. CO_2.
0.2000 " " : (Carius) 0.3158 gm. AgBr.

$C_{20}H_{32}O_2Br_8$. Calculated. C 25.43, H 3.42.
Found (No. 521). " 25.26, " 3.66.

This material, after recrystallization from a very large volume of toluene was a white amorphous powder, which darkened slightly when heated above 225°C. and sintered at 250°C. Further heating charred without melting it.

Repeated attempts were made to isolate linolic tetrabromide from the bromination mother liquor, in this and several similar experiments. All of these efforts were, however, unsuccessful.

A large amount of dark, semiliquid material was recovered after evaporation of the glacial acetic acid. From it, on addition of gasoline, a small amount of granular substance was deposited, consisting of impure octabromide, as indicated by its analysis.

0.1036 gm. substance: 0.0354 gm. H_2O and 0.1056 gm. CO_2.
0.2022 " " : (Carius) 0.3046 gm. AgBr.
Found (No. 507). C 27.79, H 3.82, Br 64.11.

The mother liquor was concentrated to dryness *in vacuo*, and the residue dissolved in absolute methyl alcohol. Powdered zinc was added, the solution heated, and hydrogen chloride passed through until a vigorous reaction was established. After 3 hours, the reaction liquor was diluted with water, and the precipitated oil extracted with ether. The residue, after evaporation of the solvent was dissolved in acetic acid, and again brominated. In this way, an additional 5 gm. of octabromide was separated, but no tetrabromide could be isolated. This bromide analyzed as follows:

0.1042 gm. substance: 0.0390 gm. H_2O and 0.0962 gm. CO_2.
0.2030 " " : (Carius) 0.3216 gm. AgBr.
Found (No. 541). C 25.17, H 4.18, Br 67.42.

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UNSATURATED FATTY ACIDS OF BRAIN LECITHINS.

BY P. A. LEVENE AND IDA P. ROLF.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 10, 1922.)

In previous publications from this laboratory it was shown that the mixed lecithins from the liver[1] and from the egg yolk,[2] yielded on hydrolysis, in addition to oleic acid, acids with more than one double bond. From the egg lecithin, linolic and arachidonic acids were isolated; whereas from liver lecithins, arachidonic acid only was obtained. The fact that linolic acid has not as yet been isolated from the liver lecithins does not prove its absence, since neither the isolation of oleic nor that of arachidonic acid is quantitative.

The present note records the results of the analysis of brain lecithins as regards the character of their unsaturated fatty acids. It was found that the brain lecithins also contain besides oleic acid, acids with more than one double bond. Of these arachidonic acid was isolated in the form of its octobromo derivative.

For the present linolic acid could not be isolated. However, the question of its presence or absence in brain lecithins is not definitely answered.

EXPERIMENTAL.

Lecithin cadmium chloride was prepared free from amino-containing impurities by the method previously described.[3] All samples of amino-free cadmium salts of brain lecithin had an iodine value not greater than 45. The following analyses are typical.

No. 404. 0.2757 gm. substance absorbed 0.1238 gm. iodine, corresponding to an iodine number of 44.

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[1] Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1922, li, 285.

[2] Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1922, li, 507.

[3] Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1921, xlvi, 353.

No. 401. 0.2582 gm. substance absorbed 0.1071 gm. iodine, corresponding to an iodine number of 41.

These salts were hydrolyzed in the usual manner, and the iodine numbers of the whole mixed fatty acids determined.

0.2663 gm. substance absorbed 0.1261 gm. iodine, corresponding to an iodine number of 47.

The fractionation of these acids, dependent on the varying solubilities of the barium and lead salts, was carried out by the method described in the preceding paper.

The acid of lower unsaturation melted at 14°C. and had an iodine number of 87.

0.2616 gm. substance absorbed 0.2259 gm. iodine.
The iodine number of oleic acid is 90.

The more highly unsaturated fraction of these acids was brominated in the usual manner, and the precipitated bromide purified by extraction with ether. A gray powder weighing 1.5 gm. was obtained. When heated to 250°C., after very gradual darkening, it sintered without melting and analyzed as follows:

0.1020 gm. substance: 0.0302 gm. H_2O and 0.0932 gm. CO_2.
0.2020 " " : (Carius) 0.3190 gm. AgBr.

$C_{20}H_{32}O_2Br_8$. Calculated. C 25.43, H 3.42, Br 67.72.
Found. " 24.91, " 3.31, " 67.22.

No linolic tetrabromide could be isolated from the mother liquor.

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ON A POSSIBLE ASYMMETRY OF ALIPHATIC DIAZO COMPOUNDS. III.

BY P. A. LEVENE AND L. A. MIKESKA.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 10, 1922.)

In previous publications,[1,2] several observations were reported which seemed to point towards the existence of an optically active diazodiethyl succinate. The present note contains an additional proof in the same direction. Curtius[3] has shown that acting with diazoethyl acetate on benzoic acid, benzoylethyl glycolate is formed. It was expected that under similar conditions diazodiethyl succinate will form benzoyldiethyl malate. It was also expected that this substance could be prepared free from malic or fumaric esters which may form as by-products, since the former possesses a much higher boiling point. These expectations were realized. Acting with diazodiethyl succinate on benzoic acid, pure benzoyldiethyl malate was obtained with an optical activity.

$$[\alpha]_D^{20} = +0.22°$$

Under similar conditions diethyl malate acting on benzoic acid did not give even a trace of benzoyldiethyl malate.

Conversion of Diazodiethyl Succinate into Benzoyldiethyl Malate.—Crude diazodiethyl succinate (45 gm.) containing 9.23 per cent diazo nitrogen, was added in small portions to 30 gm. of melted benzoic acid. The temperature of the reaction mixture was maintained at 140°C., until the reaction was complete, which required about 8 minutes. It was then cooled, dissolved in ether, and washed three times with sodium carbonate solution and finally with distilled water. After drying the ethereal solution with anhydrous sodium sulfate, the ether was removed under diminished pressure and the residue

[1] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1920–21, xlv, 593.

[2] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1922, lii, 485.

[3] Curtius, T., *J. prakt. Chem.*, 1888, xxxviii, 427.

extracted with petroleum ether to insure the removal of traces of benzoic acid. The residue was then fractionated under a pressure of about 135 mm. The first fraction boiled between 65 and 70°. The temperature then rose rapidly and the second fraction was collected between 130 and 149°C. The third fraction distilled between 150 and 160°C. The third fraction was redistilled under a pressure of 3 mm., and separated into two fractions, the first boiling at 146–147°C. and the second which was identified by analysis as benzoyl malate, boiled at 147–148°C.

0.1024 gm. substance: 0.0624 gm. H_2O and 0.2300 gm. CO_2.

$C_{18}H_{18}O_6$.	Calculated.	C 61.23, H 6.16.
	Found.	" 61.25, " 6.81.

The substance had the following rotation:

$$[\alpha]_D^{20} = \frac{+0.22° \times 100}{1 \times 100} = +0.22°$$

The optical rotation of the original diazo compound was:

$$[\alpha]_D^{20} = \frac{+1.14° \times 100}{1 \times 100} = +1.14°$$

In another experiment 30 gm. of diazodiethyl succinate were treated with benzoic acid as in the preceding experiment. A fraction, boiling at 143°C. under 0.2 mm. pressure, was obtained. It had an optical rotation of

$$[\alpha]_D^{20} = \frac{+0.12° \times 100}{1 \times 100} = +0.12°$$

The percentage of carbon and hydrogen found, agreed well with the theoretical values.

0.1014 gm. substance: 0.0604 gm. H_2O and 0.0227 gm. CO_2.

$C_{18}H_{16}O_6$.	Calculated.	C 61.23, H 6.16.
	Found.	" 61.27, " 6.61.

The original diazo compound had an optical rotation of

$$[\alpha]_D^{20} = \frac{+0.75° \times 100}{1 \times 100} = +0.75°$$

Action of Benzoic Acid on Diethyl Malate.—Malic ester (10 gm.) was gradually added to 10 gm. of melted benzoic acid exactly as in the preceding experiment. The temperature was maintained at 140–145°C. for 10 minutes. The excess of benzoic acid was removed by washing the ethereal extract with a solution of sodium carbonate. The extract was then dried, the ether removed, and the residue distilled. The entire amount distilled at 78–80°C. leaving only a trace of residue. In this case only about 1 gm. of substance was obtained whereas under similar conditions 45 gm. of diazodiethyl malate yield 42 gm. of combined distillates.

0.1012 gm. substance: 0.0716 gm. H_2O and 0.1824 gm. CO_2.

$C_8H_{14}O_5$. Calculated. C 50.52, H 7.36.
Found. " 49.15, " 7.91.

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SURFACE TENSION OF SERUM.

III. RECOVERY AFTER LOWERING BY SURFACE-ACTIVE SUBSTANCES.

BY P. LECOMTE DU NOÜY, Sc.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATE 2.

(Received for publication, February 16, 1922.)

I.

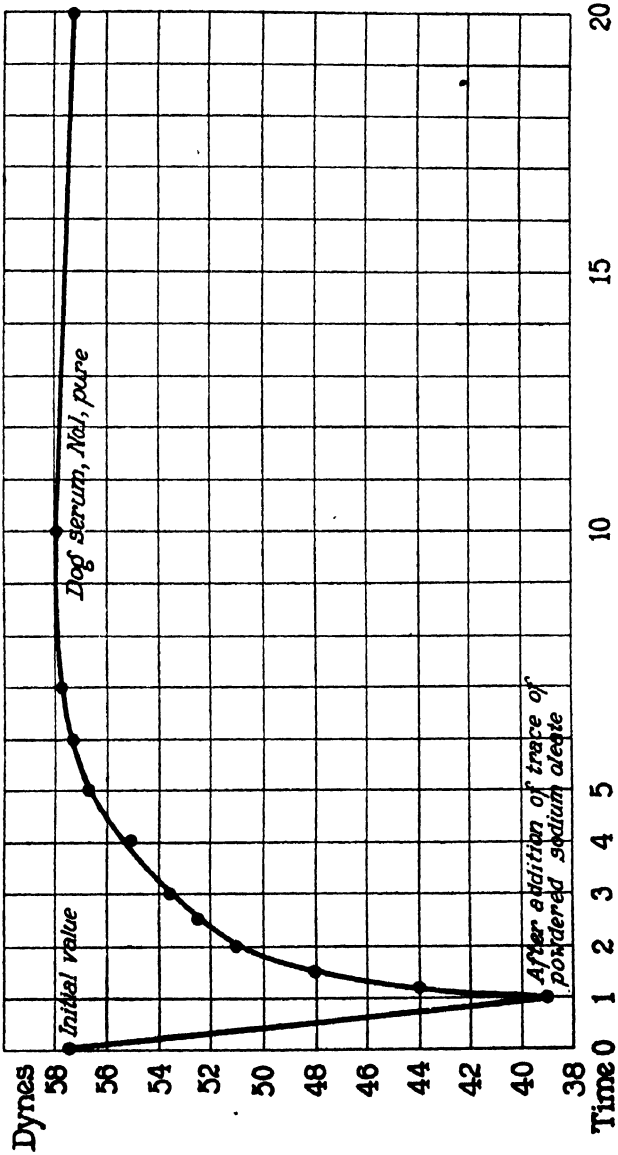
INTRODUCTION.

It has been shown in previous papers[1,2] that surface tension of serum and of solutions of serum varies in function of the time, and that the lowering of the surface tension seems to follow the laws of adsorption in the surface layer. The assumption was made that some of the constituents of the serum having a strong action upon the surface tension of water were actually adsorbed in function of time, or that certain changes in the arrangements of the group molecules took place in the surface layer.

Comparative experiments made with certain surface-active organic compounds, such as sodium oleate, glycocholate, and saponin, showed similar results.[2] But when sodium oleate, for instance, was added to serum or to a solution of serum, a converse phenomenon happened. A very considerable drop occurred first, immediately followed by a rise, which was function of the dilution of the serum and, in the case of pure serum, raised the surface tension to its normal value in a few minutes (Text-fig. 1). In other words, the action of sodium oleate was immediately counteracted and, in certain cases, entirely inhibited by an antagonistic or buffing action due to certain constituents of the serum. This explains why in jaundice large quantities of sodium

[1] du Noüy, P. L., *J. Exp. Med.*, 1922, XXXV, 575.

[2] du Noüy, P. L., *J. Exp. Med.*, 1922, XXXV, 707.



TEXT-FIG. 1. Rise of surface tension of pure serum after a drop due to the addition of sodium oleate.

glycocholate and taurocholate are present in the blood without producing any hemolysis. The same amount dissolved in saline solution would bring forth a strong lowering of surface tension and would hemolyze red cells powerfully. The purpose of this paper is the study of this phenomenon.

II.

EXPERIMENTAL.

The technique used was described in detail in the previous papers.[1,2] It will suffice to say that the measurements of the surface tension of the same layer of liquid were made by means of the du Noüy tensiom-

TABLE I.

Rise of Surface Tension of Serum in Function of Time after a Drop Due to the Addition of Sodium Oleate.

Pure Dog Serum, No. 1 (Text-Fig. 1).

Temperature 22°C.

About 1/10,000 in weight of powdered sodium oleate was used.

Time.	Surface tension.
	dynes
Before addition of sodium oleate	57.5
After " " " "	39.0
" 15 sec.	44.0
" 30 "	48.0
" 1 min.	51.0
" 1.5 "	52.5
" 2 "	53.5
" 3 "	55.0
" 4 "	56.8
" 5 "	57.3
" 6 "	57.6
" 9 "	58.0
" 20 "	57.6

eter. As usual, the utmost precautions were taken as to the cleanness of the glassware (watch-glasses, test-tubes, pipettes, stirring rods), which was washed in soda solution, then boiled for 30 minutes in a sulfuric acid and sodium dichromate cleaning fluid. Fresh dog and rabbit sera were used. When no graduated pipettes were needed, the

pipettes were chosen with very nearly the same capacity, measured by pressing evenly on the rubber nipple (1.8 to 1.9 cc.).

Immediately after the first measurement, a certain amount of sodium oleate was added and the surface tension measured. From 30 to 60 seconds elapsed between the two measurements. Although sodium glycocholate and taurocholate act in the same way, sodium oleate was chosen on account of its more marked action on surface tension. These measurements were made at regular intervals, generally 1 minute, at the beginning; in certain cases of very rapid recovery (pure serum), they were made at intervals of 15 seconds

TABLE II.

Rise of Surface Tension of Serum in Function of Time after a Drop Due to the Addition of Sodium Oleate.

Pure Dog Serum, No. 2 (Text-Fig. 2).

Temperature 22°C.

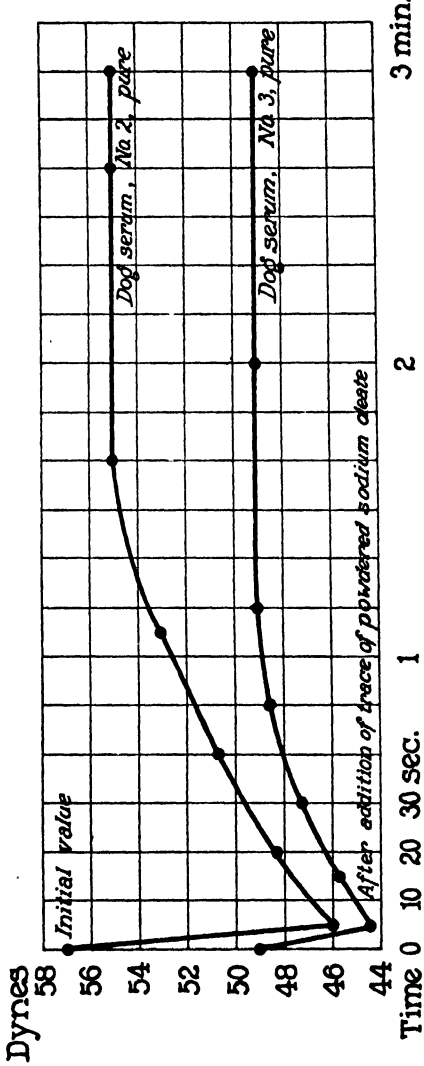
About 1/10,000 in weight of powdered sodium oleate was used.

Time.	Surface tension.
	dynes
Before addition of sodium oleate	56.0
30 sec. after addition of sodium oleate	46.0
After 1 min.	53.0
" 1.5 "	55.0
" 2.5 "	55.0

during the first 1 or 2 minutes. In order to avoid experimental errors, all the measurements were repeated, in certain cases four times.

1. Pure Serum.—The results obtained with Serum 1 are shown in Table I. This serum had previously been heated at 56°C. for 2 hours and did not manifest any sign of spontaneous lowering of surface tension when exposed in a watch-glass. After 9 minutes the value of the surface tension was higher than the initial value. An attempt to explain this fact will be made in a later paragraph. With Serum 2 (Table II and Text-fig. 2) the final surface tension was 1 dyne lower than the initial value.

With Sera 1, 2, and 3 (Tables I to III), the rise was extremely rapid, and after a drop of 18.5 dynes, the initial value was reached in 5



TEXT-FIG. 2. Rise of surface tension of pure serum after a drop due to the addition of sodium oleate.

minutes in the first experiment; in the second, the drop being much smaller (10 dynes), the initial value was reached in 1½ minutes, and in the third case (4.5 dynes), in 80 seconds. The same amount of sodium oleate added to saline solution brought its surface tension down to about 32.5 dynes permanently. Every serum seemed to react in its own specific way as far as time and final value were concerned.

The result obtained by the addition of 1 drop of sodium oleate solution at a concentration of 1/100 is given in Table IV and Text-fig. 3. The final concentration of sodium oleate in the solution was about 1/12,000. The concentration of sodium oleate was much smaller and

TABLE III.

Rise of Surface Tension of Serum in Function of Time after a Drop Due to the Addition of Sodium Oleate.

Pure Dog Serum, No. 3 (Text-Fig. 2).

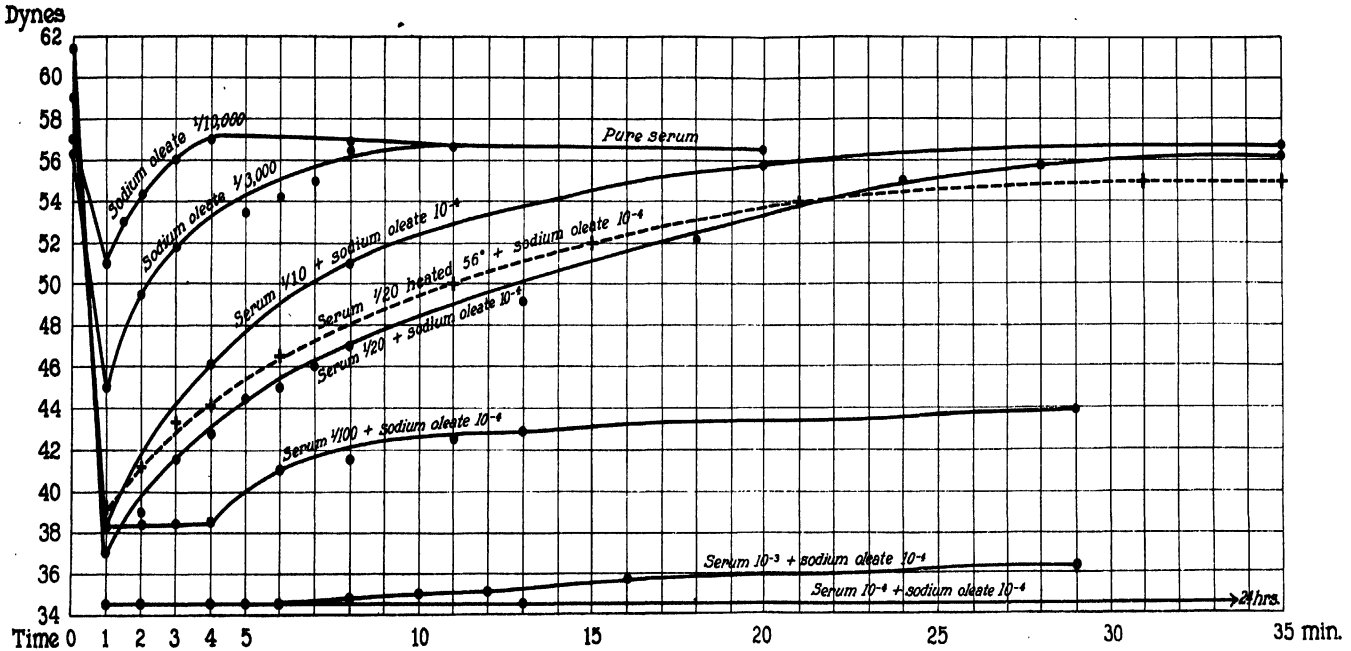
Temperature 22°C.

About 1/10,000 in weight of powdered sodium oleate was used.

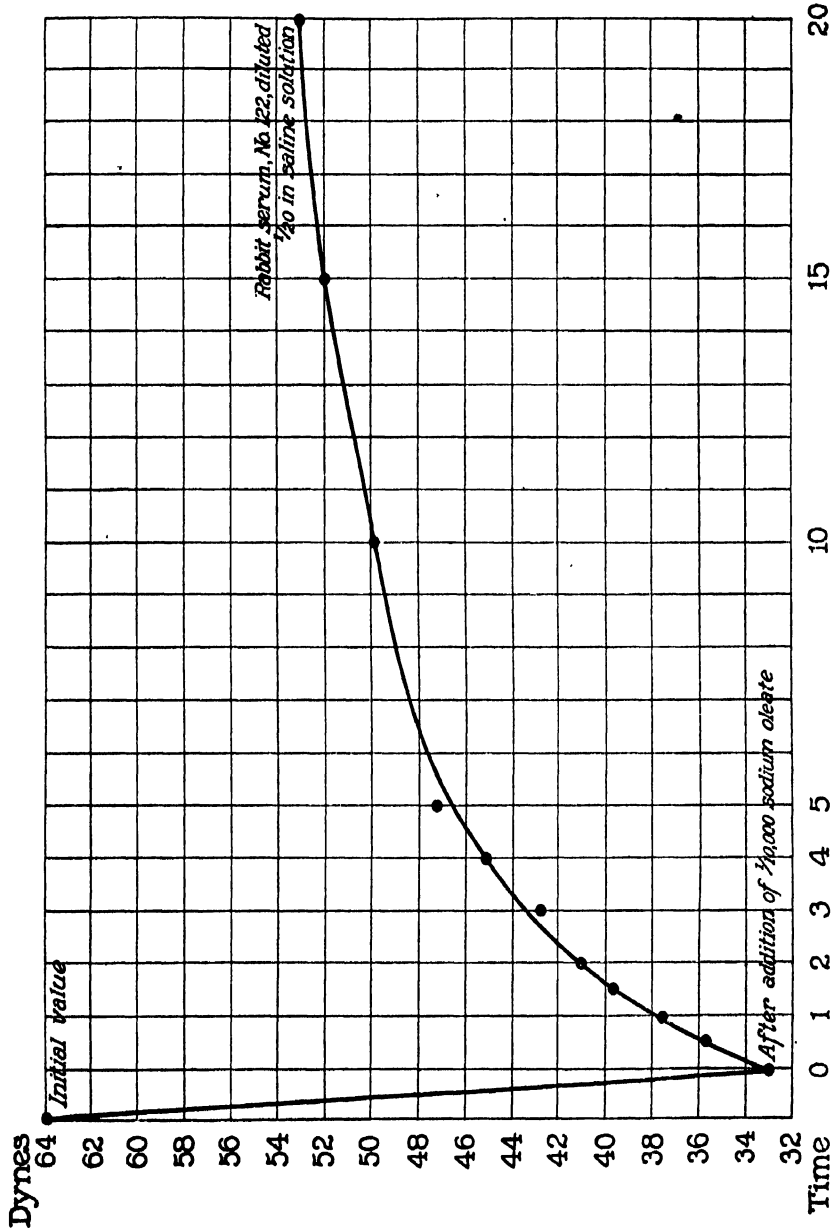
Time.	Surface tension.
	dynes
Before addition of sodium oleate	49.0
30 sec. after addition of sodium oleate	44.5
After 40 sec	47.2
" 1 min	48.5
" 1.3 "	49.0

the drop in surface tension was reduced accordingly. In Table V the same experiment was made, but 3 drops of the same sodium oleate solution were added instead of 1, making the concentration of the surface-active substance about 1/4,000. The final surface tension is equal to the initial value. It sometimes happens that after reaching its initial value rapidly, a drop occurs, due to the normal phenomenon studied in the preceding papers.[1,2]

2. Dilutions of Serum.—Dilutions of serum in saline solution at 0.9 per cent were prepared at concentrations of 1/10, 1/20, 1/100, 1/1,000, and 1/10,000. The same amount of sodium oleate was added to the same amount of solution. For these measurements, a 2 cc. graduated pipette was used. The average weight of a drop



TEXT-FIG. 3. Rise of surface tension of pure serum and serum solutions after a drop due to the addition of sodium oleate.



TEXT-FIG. 4. Rise of surface tension of a 1/20 serum solution after the addition of 1/10,000 of sodium oleate.

reading was still 35.6. This phenomenon is therefore quite different from that of the decrease in surface tension of serum solutions, which showed a maximum activity in most cases at a dilution of about 1/10,000.

The results in percentage rise and in absolute rise in 30 minutes are expressed by Text-figs. 5 and 6. For the same dilutions, the time

TABLE VI.

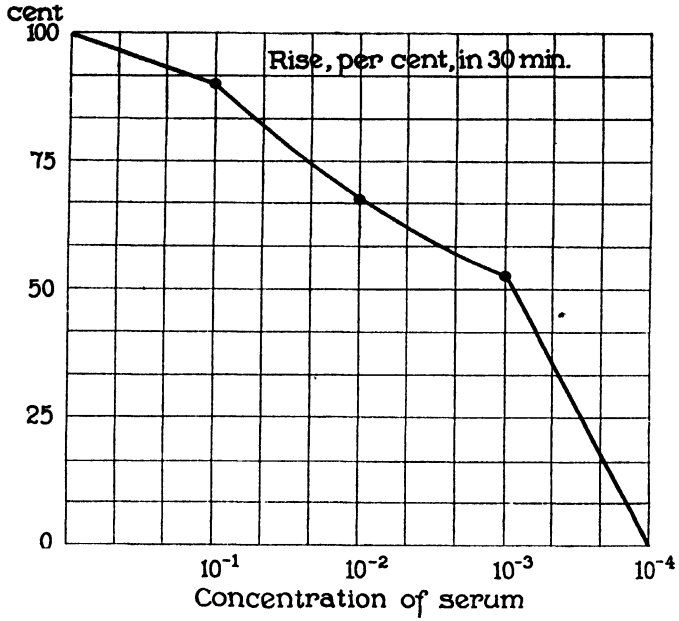
Rise of Surface Tension of Serum Solutions in Function of Time after a Drop Due to the Addition of Sodium Oleate.

Rabbit Serum, No. 121, Diluted in Isotonic NaCl Solution (Text-Fig. 3). Temperature 22°C.

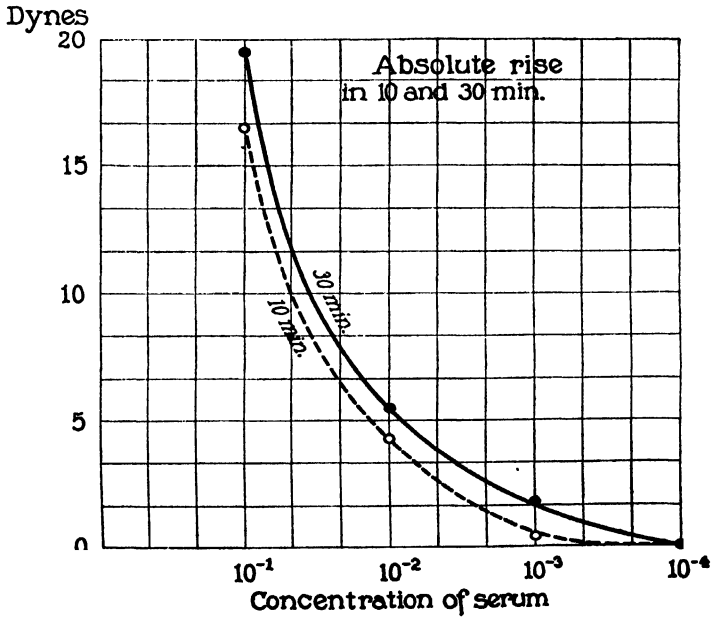
Sodium oleate, 10^{-4}, was used.

Time.	Surface tension. Concentration of serum.				
	1/10	1/20	1/100	1/1,000	1/10,000
	dynes	*dynes*	*dynes*	*dynes*	*dynes*
Before addition of sodium oleate.....	62.5	62.0	64.0	69.5	72.0
After " " " "	38.5	37.0	38.4	34.5	35.6
" 1 min.....		39.0	38.5	34.5	35.6
" 2 "	46.0	41.5	38.5	34.5	35.6
" 3 "		42.8		34.5	35.6
" 4 "	48.5	44.4	40.0	34.6	35.6
" 5 "		45.0	41.0	34.7	
" 6 "	51.0	46.0	41.7	34.8	
" 7 "		47.0			35.6
" 8 "	55.9			35.0	
" 9 "			42.6	35.2	
" 15 "	55.0	51.0	43.3	35.7	35.6
" 30 "	56.5	56.0	44.0	36.5	35.6

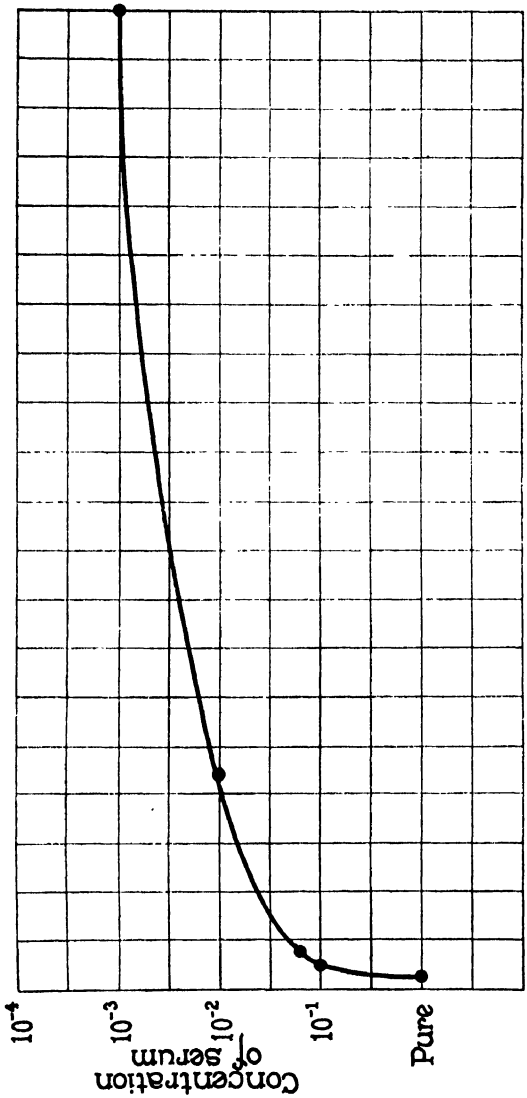
necessary to raise the surface tension of the solutions by 2 dynes is represented by Text-fig. 7. It will be seen that, by diluting the serum tenfold, the time is only doubled, while it is increased seventeenfold by diluting the serum solution again ten times; that is, to 1/100. Doubling the dilution at a concentration of 1/10, *viz.* 1/20, has the same effect as diluting pure serum tenfold. These phenomena, although difficult to interpret at present, are very probably due to the fact that surface tension is governed by the best possible arrange-



TEXT-FIG. 5. Percentage rise of surface tension of serum solutions and serum in function of the concentration of the serum after the addition of the same amount of sodium oleate. Time 30 minutes.



TEXT-FIG. 6. Rise of surface tension of serum solutions in function of the concentration of serum after the addition of the same amount of sodium oleate. Time 30 minutes.



TEXT-FIG. 7. Time necessary to raise the surface tension of serum solutions by 2 dynes after the addition of the same amount of sodium oleate.

ment of group molecules in the surface layer, as was stated in previous papers. The explanation of the broken aspect of the curve in Text-fig. 5 lies in the better knowledge of the structure of the

TABLE VII.

Quantitative Determination of the Antagonistic Action in Function of Time of a 1/10 Serum Solution.

Rabbit Serum, No. 120 (Text-Fig. 8).

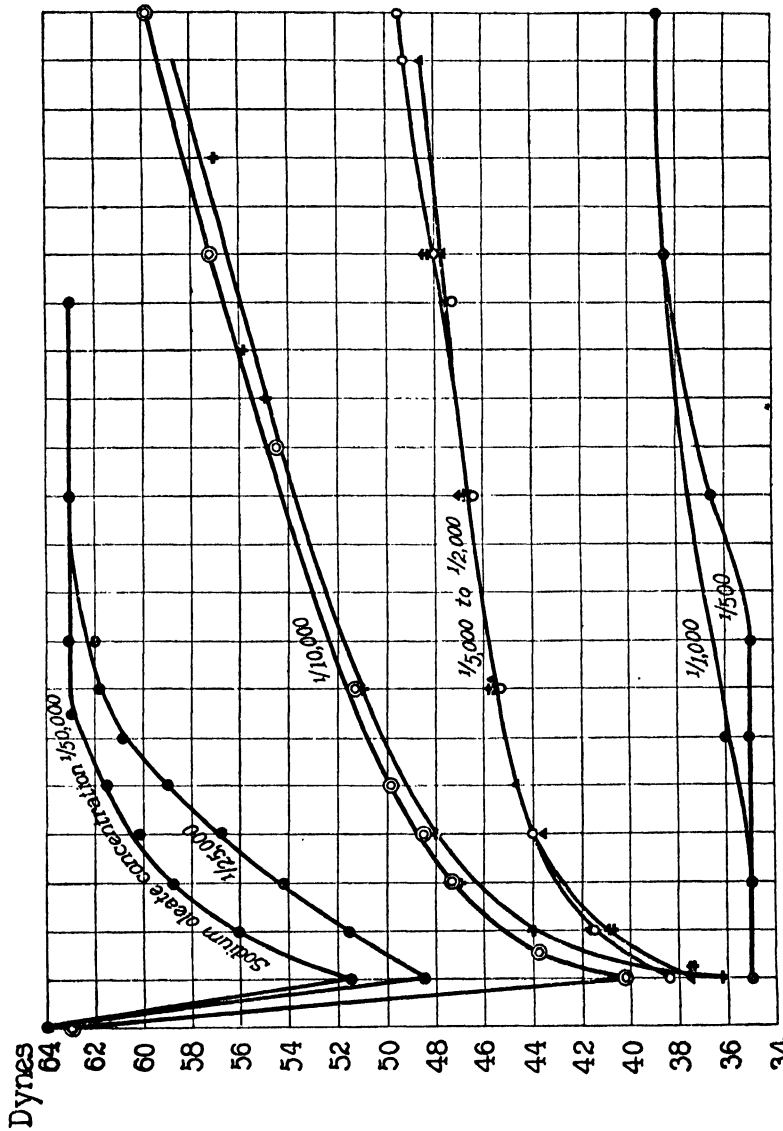
Temperature 22°C.

In every case, different amounts of sodium oleate were added to 1.9 gm. of serum solution. Each drop of sodium oleate weighed 0.019 gm.; 1, 2, 3, or more drops were added. Isotonic saline solution was used for the dilutions.

Time.	Surface tension. Concentration of sodium oleate. 1/50,000	1/25,000	1/10,000	1/5,000	1/2,500	1/2,000	1/1,000	1/500
	dynes	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*
Before addition of sodium oleate.....	63.0	63.0	62.5	62.6	62.5	61.2	63.0	63.0
After " " " "	51.5	48.5	40.2	38.4	36.5	36.5	34.3	34.2
" 1 min.....	56.0	51.6	46.5	41.5	40.9	41.5	34.3	34.5
" 2 "	58.5	54.2	47.3	43.0	42.9	42.9	34.5	34.5
" 3 "	60.2	57.0	48.5	44.0	44.0	43.7	34.5	34.5
" 4 "	61.5	59.0	49.8	44.5		44.6	34.0	
" 5 "	63.0	60.8	50.5	44.7	45.0	45.0	36.0	
" 6 "	63.2	61.8	51.3		45.4	45.4		
" 7 "						45.8		
" 8 "		62.2						
" 10 "	61.0	63.0		46.4	46.6	46.6		
" 11 "			54.5					
" 13 "								38.6
" 15 "		61.0		48.0	48.0	47.5	38.4	38.6
" 16 "			57.9					
" 21 "			57.9					
" 30 "			61.0	51.6				
" 1 hr., 30 min.....			58.0	52.3			39.5	39.2

protein and other colloidal substances of the serum, and of their physical behavior when placed in contact with other free molecules.

In order to study the process of recovery of surface tension of serum solutions quantitatively, varying amounts of sodium oleate in solution were added to the same quantity of serum solution at 1/10 dilution. The results are given in Table VII and Text-fig. 8.



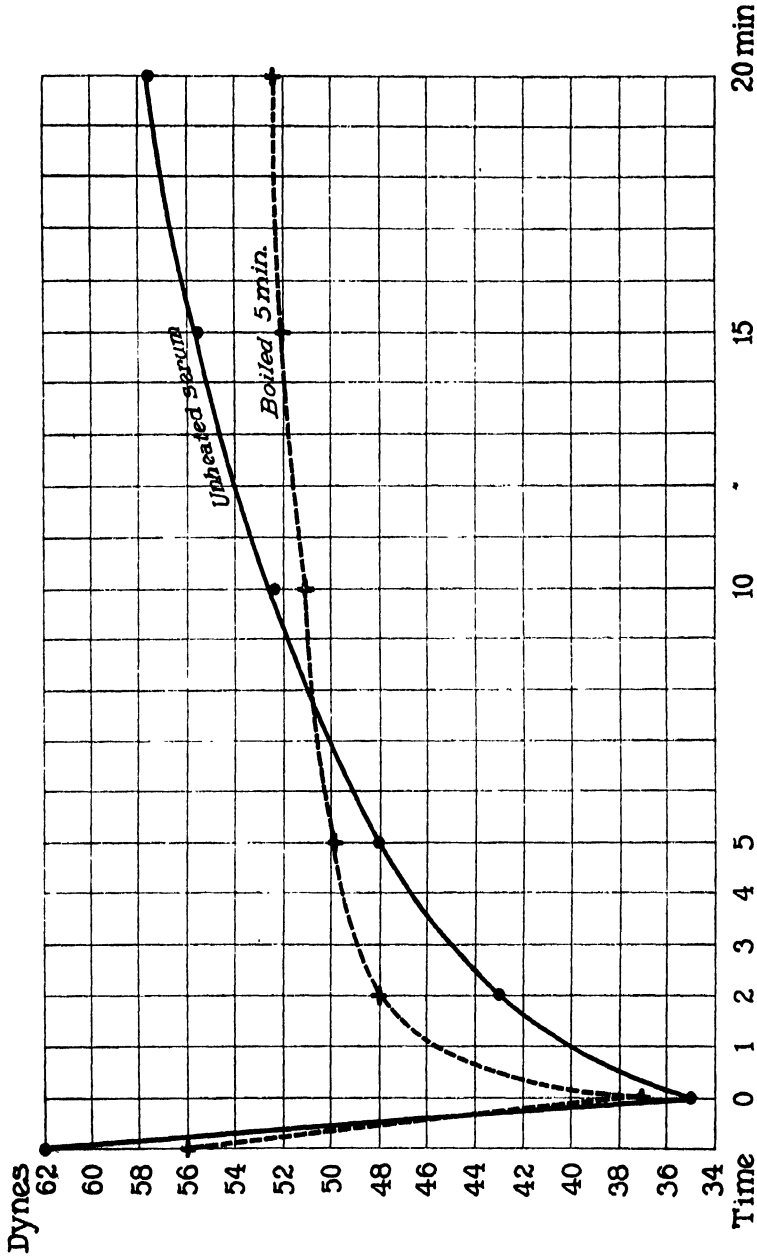
TEXT-FIG. 8. Rise of surface tension of a 1/10 serum solution after the addition of different amounts of sodium oleate

It is decidedly important whether the solution is stirred or not after the addition of sodium oleate, or whether the surface-active substance is added powdered or in a concentrated or dilute solution. In other words, the results will be different if the concentration of 1/1,000 of sodium oleate is obtained by adding 1 drop of a 1/10, or 10 drops of a 1/100 solution of sodium oleate to the serum, compensation being made for the different amount of liquid. Generally speaking, the antagonistic action of the serum is more powerful when the sodium oleate is less dilute. It is strongest for pure powdered sodium oleate.

3. Action of Heat.—Heat modifies the properties of serum profoundly, even at a relatively low temperature such as 56°C. However, although the changes are important as far as the biological properties are concerned, they are undetectable to the chemist and the physicist. Heated serum is no longer the same, but were it not for biological tests, such as the study of hemolytic power, it would be impossible to differentiate it from unheated serum. Experiments on the recovery of the surface tension of serum solutions show that generally when a serum has been heated, the beginning of the recovery is more rapid, but that after 15 or 20 minutes, sometimes less, its curve crosses that of the recovery of the unheated sample, and remains below it. The serum acts practically in the same way whether heated before or after dilution. When heated before dilution, the phenomenon is slightly more marked. It was already known that dilution had a protective action on serum. The higher the temperature, the more marked the phenomenon, whether the serum was diluted in pure water or in saline solution. An idea of this phenomenon is given in Text-fig. 9.

In order to compare the sensitivities of the physiological and of the physical method, two samples of a 1/10 serum solution in saline solution were studied from the standpoint of the lowering of surface tension after the addition of sodium oleate. One sample was boiled in a water bath for 5 minutes, the other was not. Clark, Zinck, and Evans[3] observed that, after boiling, the protective action of human serum against hemolysis of red cells (guinea pig) by sodium oleate

[3] Clark, H. M., Zinck, R. H., and Evans, F. A., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 328.



TEXT-FIG. 9. Rise of surface tension of a 1/10 serum solution before and after heating 5 minutes at 100°C.

was decreased. In other words, it required a smaller concentration of sodium oleate to produce the same degree of hemolysis (1/50,000 with fresh serum, 1/65,000 with boiled serum). We were unable to check up these results by using rabbit serum and hen cells, but our measurements showed that the surface tension of the boiled serum solution was inferior by 10 dynes to that of fresh serum, which would suffice in itself to explain a difference, and that the recovery, although more rapid in the first 8 minutes, was much below that of the fresh serum at the end (Text-fig. 9.) When using serum solution in distilled water (dilution 1/20), the action was similar (Text-fig. 10).

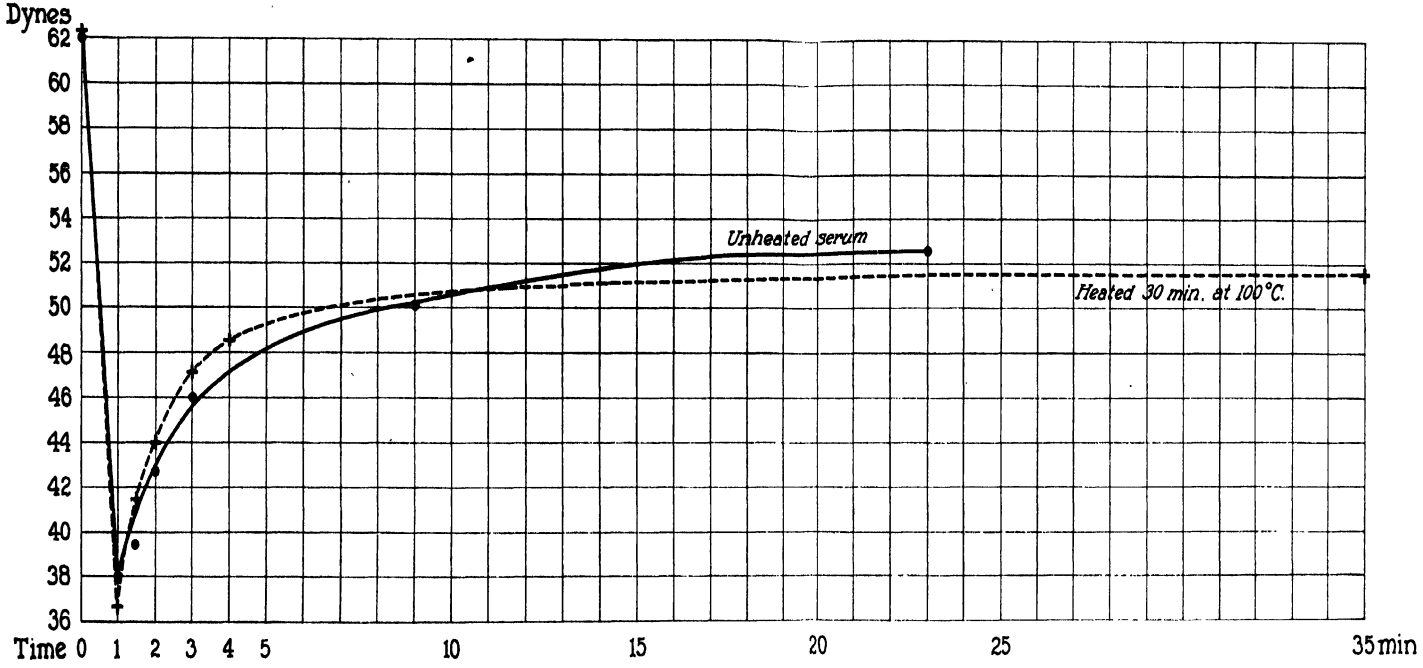
In this case, the study of the surface tension shows that the phenomenon is dependent on two factors; the initial value of the surface tension plays one part, and the antagonistic action of the serum another. The advantage of the physical method was that it decomposed a complex phenomenon into its elements, introduced the factor time, and did not depend on such an unreliable standard as hemolysis of red cells.

The considerable modifications brought about in the serum by temperature are also very strikingly illustrated by Figs. 1 to 4. These are photographs of the crystallization of four samples of a 1/10 solution of serum in saline solution, to which 1/10,000 of sodium oleate had been added. The first sample (Fig. 1) was unheated. The second (Fig. 2) was heated at 56°C. for 2 hours. The third (Fig. 3) was heated at 70°C. for 1 hour, and the fourth (Fig. 4) was boiled for 5 minutes. These phenomena will be studied in another paper.

III.

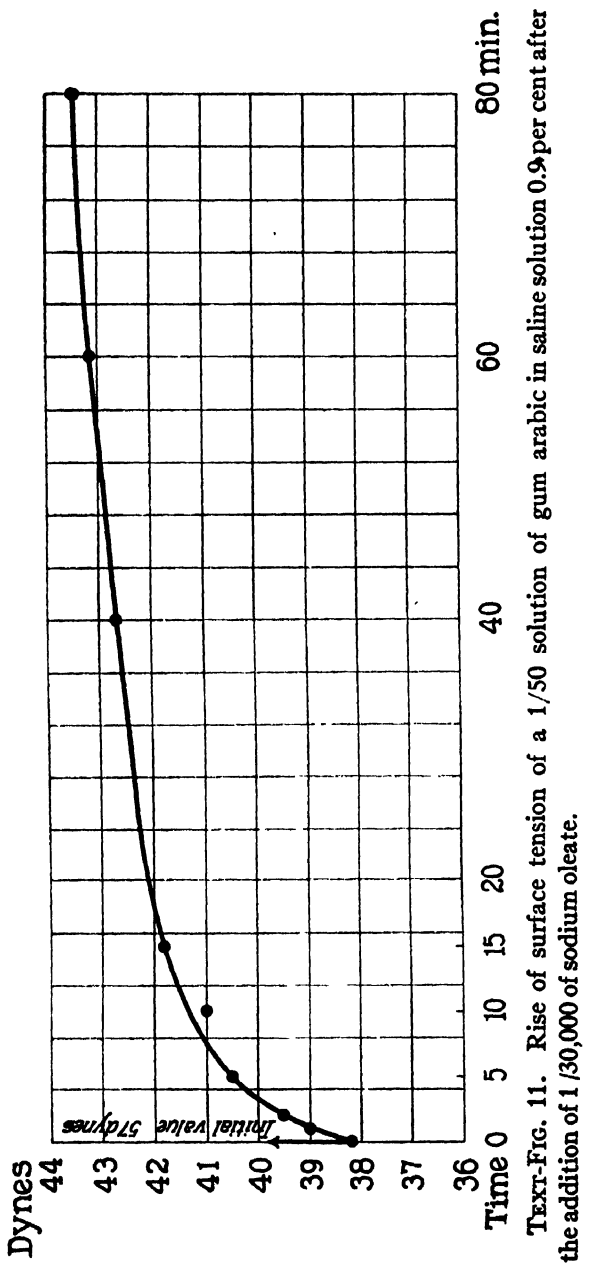
DISCUSSION.

All these facts may be accounted for by simply assuming that this phenomenon of recovery is not due to a special substance but to the adsorption of the surface-active molecules by the large colloidal micellæ of the serum. Comparison of the curves of recovery with ordinary curves of adsorption makes this plain, but an attempt was made to obtain the same phenomenon with inert colloidal solutions, such as gelatin and gum arabic. If our hypothesis

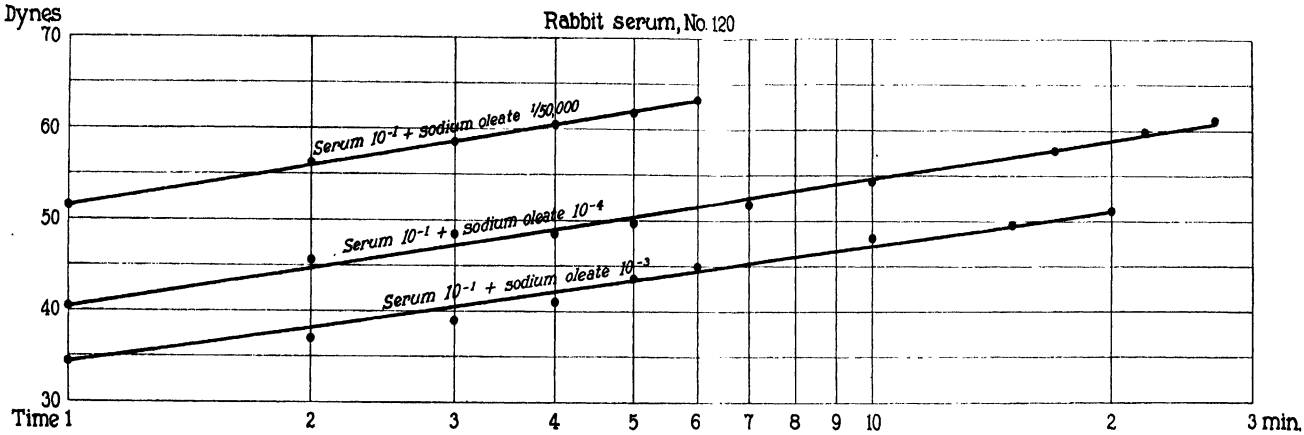


TEXT-FIG. 10. Rise of surface tension of a 1/20 serum solution in distilled water before and after heating 30 minutes at 100°C.

were correct, the same recovery should take place as soon as *any* colloidal particles should be present in an aqueous solution whose surface tension had been lowered by the addition of sodium oleate. The order of magnitude of the phenomenon depending mainly on the size of the particles, a much slower process was expected. Experiments showed the correctness of the views expressed above (Text-fig. 11), solutions of gelatin, egg albumin, and gum arabic acting in a way absolutely comparable to that of serum. When sodium oleate, powdered or in highly concentrated solution, is added to the serum, without much stirring, the oleate spreads all over the free surface, forming large aggregates of molecules which are rapidly adsorbed by the serum micellæ of the surface layer, some of them becoming heavy enough to fall to the bottom. At a certain optimum concentration, almost all the oleate molecules are being adsorbed; that is, withdrawn from the surface. They form a thick coating around the molecules or group molecules of serum, which tends to force them apart a little more than they were before. This explains why the surface tension, after recovery, often reaches a slightly higher value than before the addition of oleate. However, a great number of micellæ in the bulk are free from sodium oleate molecules and they become progressively adsorbed in the surface layer, thus lowering slightly the surface tension, while the sodium oleate-coated micellæ remain in the bulk or are precipitated, and the surface tension falls back to normal. On the contrary, should a dilute solution be used, the oleate molecules are very mobile. No large aggregates are formed, the solution mixes readily with the serum, the surface-active molecules are brought near every serum molecule in the bulk, adsorption follows, but the result is that *all* serum micellæ carry a certain amount of oleate molecules so that the recovery is slow and the surface tension may never reach as high a value as in the case in which concentrated oleate solution was used. Of course, in the case of powdered or highly concentrated oleate, stirring will produce the same effect as if the solution were more diluted. But when the solution is stirred only slightly or not at all, it is easy to observe the occurrence of a visible and rather coarse precipitate, while if the solution has been vigorously stirred, it remains clear and merely opalescent. When a solution of 1/10 of



TEXT-FIG. 11. Rise of surface tension of a 1/50 solution of gum arabic in saline solution 0.9 per cent after the addition of 1/30,000 of sodium oleate.



TEXT-FIG. 12. Rise of surface tension of a 1/10 serum solution after the addition of different amounts of sodium oleate (curves of Text-fig. 8), plotted on semilogarithmic paper.

oleate is used, films are seen floating at the surface. This never occurs with a solution of 1/100.

In our experiments, the phenomenon of recovery was not a simple function of the concentration of sodium oleate; whether the concentration of oleate was 1/5,000 or 1/2,000, the rate of recovery was almost identical at the beginning, while there was a considerable difference between the rates of recovery of solutions at 1/10,000 and 1/5,000 (Text-fig. 8). The same gap exists between 1/2,000 and 1/1,000 while 1/1,000 and 1/500 act practically in the same way. Too little is known about the structure of the serum molecules and the intimate mechanism of the surface tension to attempt a quantitative explanation of these facts. There is no doubt but that the solution of the problem will be found in the knowledge of the interactions of the group molecules in the surface layer. The curves (Text-fig. 8) correspond to possible geometrical arrangements of the group molecules, resulting in certain optima stray fields. These facts will only be cleared up some day by a thorough study of the physics of the molecules, probably on the basis of Langmuir's theory.[4]

Some of the curves of recovery of surface tension were plotted on semilogarithmic paper and appear as straight lines (Text-fig. 12). This indicates that the process follows the compound interest law, and may be expressed by an equation of the form

$$\gamma = ae^{bx}$$

similar to that established for the phenomenon of spontaneous lowering of surface tension,[1,2] and adsorption in general.

IV.

CONCLUSIONS.

1. The equilibrium of the serum corresponding to its normal minimal surface tension is as stable and difficult to break, under ordinary conditions, as the osmotic tension equilibrium. The addition of a strong surface-active substance (sodium oleate, glycocholate, or taurocholate), will not lower it definitely, unless the substance is present in large amounts and in solution. After the first rapid drop

[4] Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1848.

has occurred, a process of recovery takes place, which brings back the normal surface tension in a short time (from 2 to 6 minutes in the case of pure serum). As a drop in the surface tension of the serum of animals may be very injurious to the red cells, this process of recovery is a normal one of defense in all cases in which surface-active substances (bile) are set free in the blood.

2. When diluted, the serum shows the same phenomenon to a smaller extent; the time of recovery is very much longer and the final surface tension is always lower than the original value. At a dilution of 1/10,000, no recovery takes place, the dilution being too high to overcome the lowering action of 1/10,000 of sodium oleate.

3. The recovery is stronger when the surface-active substance is added powdered or in a highly concentrated solution, and not stirred.

4. The recovery does not seem to be inversely proportional to the concentration of sodium oleate, when added superficially. Doubling the concentration at 1/2,000, for example, gives the same curve of recovery. This happens under certain conditions; namely, when the liquid is not stirred after the addition of sodium oleate.

5. This recovery is due to a purely physical phenomenon, namely adsorption, and is not specific for the serum. Other colloidal solutions, such as gum arabic, egg albumin, gelatin, and silver and gold sols, show it, only to a smaller degree. The process of recovery follows a logarithmic law in all cases, expressed by an equation of the form

$$\gamma = ae^{bx}$$

6. Temperature affects this phenomenon. At first it enhances it, but finally decreases it. This would seem to connect the loss of the property of the serum known as complement in a serum with a modification of the physical properties of this serum. This phenomenon is being investigated further.

EXPLANATION OF PLATE 2.

FIGS. 1 to 4. Action of temperature on the crystallization of serum solutions, concentration 1/10 in saline solution 0.9 per cent (rabbit serum).

FIG. 1. Unheated.

FIG. 2. Heated at 56°C. for 2 hours.

FIG. 3. Heated at 70°C. for 1 hour.

FIG. 4. Heated at 100°C. for 5 minutes.

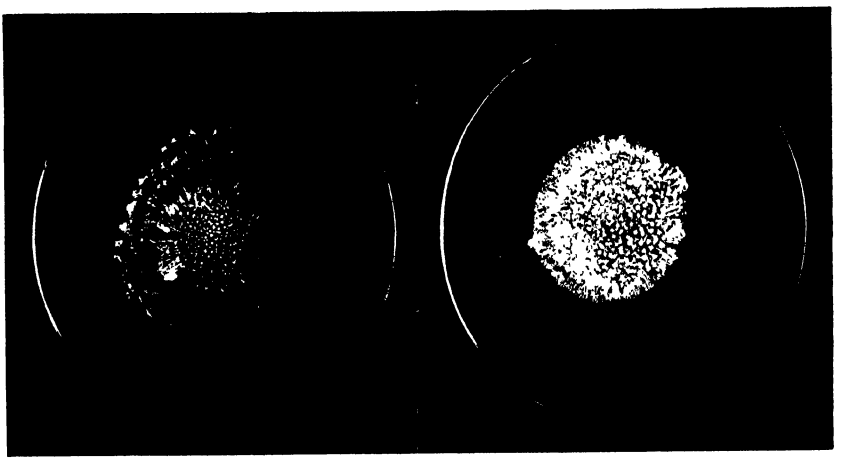


FIG. 1. FIG. 2.

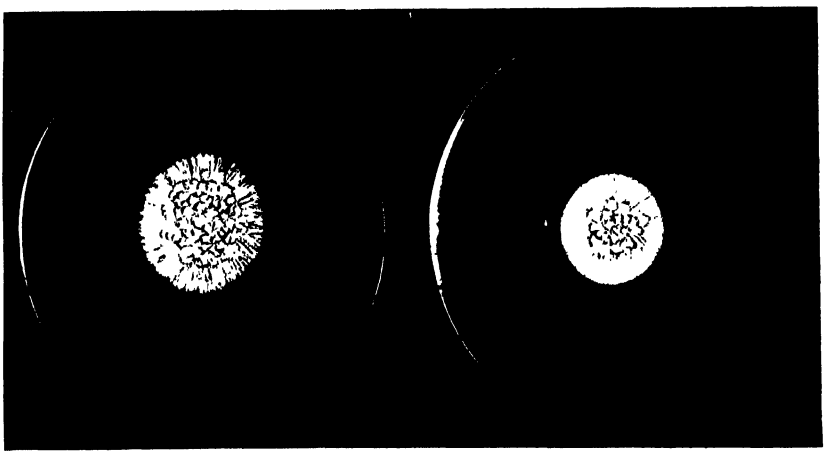


FIG. 3. FIG. 4.

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MIXED CULTURES OF PURE STRAINS OF FIBROBLASTS AND EPITHELIAL CELLS.

BY ALBERT H. EBELING, M.D., AND ALBERT FISCHER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 15 TO 18.

(Received for publication, May 5, 1922.)

For years the question of dedifferentiation or transformation of tissue cells into an indifferent embryonic cell type when cultivated *in vitro* has been under discussion. Champy[1] cultivated kidney tissue, and found that its structure changed; indifferent tubules were formed which later lost their renal and finally their epithelial characteristics. From this and other experiments, he concluded that embryonic tissue cells cultivated *in vitro* dedifferentiate sooner or later, sometimes after a few hours. Uhlenhuth[2] showed that tapetum cells from the retina changed their typical epithelial character with modifications in the consistency of the culture medium. We found also that, when epithelial cells were allowed to grow embedded in the clot, their shape changed from polygonal to fusiform. Therefore, under these conditions, cultures of epithelial cells looked like cultures of fibroblasts, but under high magnification, there was no difficulty in distinguishing epithelial cells from fibroblasts. Although the epithelial cells proliferating in the clot are spindle-shaped, they have a peculiar way of growing close to each other (pavement-like), which is not true of fibroblasts. In a previous article,[3] it was shown that epithelial cells in pure cultures still remained typical after more than 3 months. The morphological characteristics of the cells did not change as long as the strain was kept under constant cultural conditions; that is, on the free surface of the plasma clot under a film of embryonic tissue juice.

[1] Champy, C., *Bibliog. Anat.*, 1913, xxxiii, 184.
[2] Uhlenhuth, E., *J. Exp. Med.*, 1915, xxii, 76.
[3] Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367.

In order to settle the question of dedifferentiation, it was thought of interest to determine whether epithelial cells and fibroblasts could be distinguished from one another after they had been allowed to grow side by side in the same culture for several generations.

I.

Technique.

Fragments of the strains of fibroblasts and epithelium were cultivated side by side under identical conditions (Fig. 1). After 48 hours, a distinct difference was observed in the character of the growth of the two fragments. The epithelium grew in a compact mass, with the individual cells in close contact. The fibroblasts migrated into the culture medium and formed a network. In the fixed preparations stained with methylene blue, it was not possible to determine definitely whether any amalgamation of the two cell types took place after the growth from both fragments had united. No more striking result was obtained by the Van Gieson method. Then, fragments of a 2 month old strain of epithelium and a 10 year old strain of fibroblasts were cultivated together for several generations. After a few passages, the fibroblasts overgrew the epithelial fragment, covering it completely. The combined culture showed a peripheral growth composed of fibroblasts, and the only apparent indication of the presence of epithelium was that the central portion of the culture appeared semitransparent and homogeneous, and not as dense and opaque as a typical culture of pure fibroblasts which had not been divided for the same number of generations. At this stage, the mixed cultures were divided and subcultures made. These in turn were allowed to grow for 48 hours, and were again divided through the central portion. This procedure was continued for seven generations and then the preparations were fixed, sectioned, and stained by Van Gieson's method. The sections showed typical epithelial and connective tissues, as found in the organism. The epithelium appeared greenish yellow in contrast with the connective tissue, which appeared pink and contained many fibrillæ which were stained a marked pink. No parts of the section showed an amalgamation of the two cell types. The epithelial cells were everywhere distinctly

differentiated. In many places a definite structural arrangement of the cell elements was observed (Fig. 2). The epithelial cells had grouped themselves to form tubules with distinct lumina. In several of the sections, the lumina could be seen filled with a homogeneous colloidal secretion (Fig. 3). The arrangement of the epithelial cells forming the tubules resembled the conformation found in sections of salivary glands. The individual cells which formed the tubules had their nuclei disposed close to the basal membrane.

In some parts of the section, epithelial cells could be seen penetrating the surrounding layer of fibroblasts and appearing on the free surface of the tissue fragment. In other parts, large masses of amorphous material (dead epithelial cells and secretions), surrounded by a layer of low epithelium, could be observed (Fig. 2).

II.

DISCUSSION.

These experiments show that epithelium cultivated for 2 months *in vitro* retained its morphological characteristics which differed decidedly from those of fibroblasts. But a still more striking fact was observed; namely, that the differential Van Gieson stain brings out the chemical difference between the two cell types when they are allowed to grow together. The epithelium was observed to have formative ability; *i.e.*, the epithelial cells arrange themselves in winding tubules. This has already been mentioned in an earlier report of experiments[3] in which the epithelial cells, cultivated on the free surface of the clot, grew in a single layer and were described as organizing themselves in structures which closely resembled cross-sections of glands. The experiments herein described confirm this statement, since the tubular arrangement may be followed throughout serial sections.

Champy[1] states that no strains of cells can be cultivated for any length of time *in vitro* without a change occurring in their morphological characteristics. We have shown that fibroblasts[4] and epithelial cells[3] may be cultivated in pure cultures for long periods without dedifferentiation. Therefore, in this case it seems that Champy's

[4] Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755.

statement does not confirm the experimental data. On the other hand, he also states that epithelial cells in the presence of connective tissue cells do not dedifferentiate. This fact is fully substantiated by the present work.

The foregoing experiments have made it possible to analyze more accurately the character of the growth of epithelium *in vitro*. When the fragment is cultivated upon the surface of a clot, the growth is characterized by the formation of a membrane, the individual cells are polygonal, and their growth resembles a pavement epithelium. This is the typical formation obtained by surface cultivation. When the fragment is cultivated in the clot, the growth seems to depend upon the existing condition within the medium and the disposition of the embedded fragment. When the cells are allowed to invade the medium uniformly, an extensive membrane is formed, very much like that obtained in surface growth (Fig. 4), but the individual cells are spindle-shaped (Fig. 5) and not polygonal. If the condition of the coagulum prevents the uniform outgrowth of new cells from the mother fragment, then the cell invasion is characterized by the formation of branching tubules of various forms, but essentially the arrangement of the growing cells is such as to form a more or less organized structure resembling hollow tubes (Fig. 6). The growth and migration of epithelial cells seem to be much more dependent upon the mechanical conditions than those of fibroblasts.

When growth occurs in membrane formation, it is rapid and extensive. When the tubular type results, the rate of growth is markedly slower and the actual increase in mass is small, although the length of the tubular outgrowth may be extensive.

Now that it has been proved that epithelium and fibroblasts cultivated *in vitro* remain two different types of cells, with individual characteristics, innumerable experimental possibilities are opened. It is obvious that the study of their respective interactions under different experimental conditions will lead to interesting findings.

III.

CONCLUSIONS.

1. Strains of epithelium and fibroblasts cultivated side by side in the same medium keep their individual characteristics. When sec-

tioned and stained by the Van Gieson method, the cultures show the epithelium stained greenish yellow and the fibroblasts and their fibrillæ pink.

2. There are no transition forms between the epithelial cells and fibroblasts.

3. The epithelial cells belonging to an older strain are still able to form primitive structures of winding tubules, with typical glandular epithelium.

4. Under the conditions of the experiments, no dedifferentiation takes place.

EXPLANATION OF PLATES.

PLATE 15.

FIG. 1. Experiment 1532–4. Culture of fibroblasts and epithelial cells cultivated together, after 48 hours incubation. Stained with methylene blue. *A*, fragment of a 10 year old strain of fibroblasts; *B*, fragment of a 2 month old strain of epithelium. × about 20.

FIG. 2. Experiment 1387–1. Section through a mixed culture of a 10 year old strain of fibroblasts and 2 month old strain of epithelium. The preparation was fixed and stained by Van Gieson's method after having undergone seven passages *in vitro*. *A*, glandular arrangement of epithelial cells surrounded by fibroblasts; *B*, network of fibroblasts and bundle of fibrillæ; *C*, an area of degenerated epithelial cells surrounded by low epithelium. × 120.

PLATE 16.

FIG. 3. Experiment 1387–1. Another section through the same culture as Fig. 2. *A*, glandular arrangement of epithelium; *B*, lumina; *C*, a lumen filled with secretion; *D*, waved connective tissue fibrillæ; *E*, characteristic position of the nucleus in the cell as it appears in secretory epithelium. Stained by Van Gieson's method. × 400.

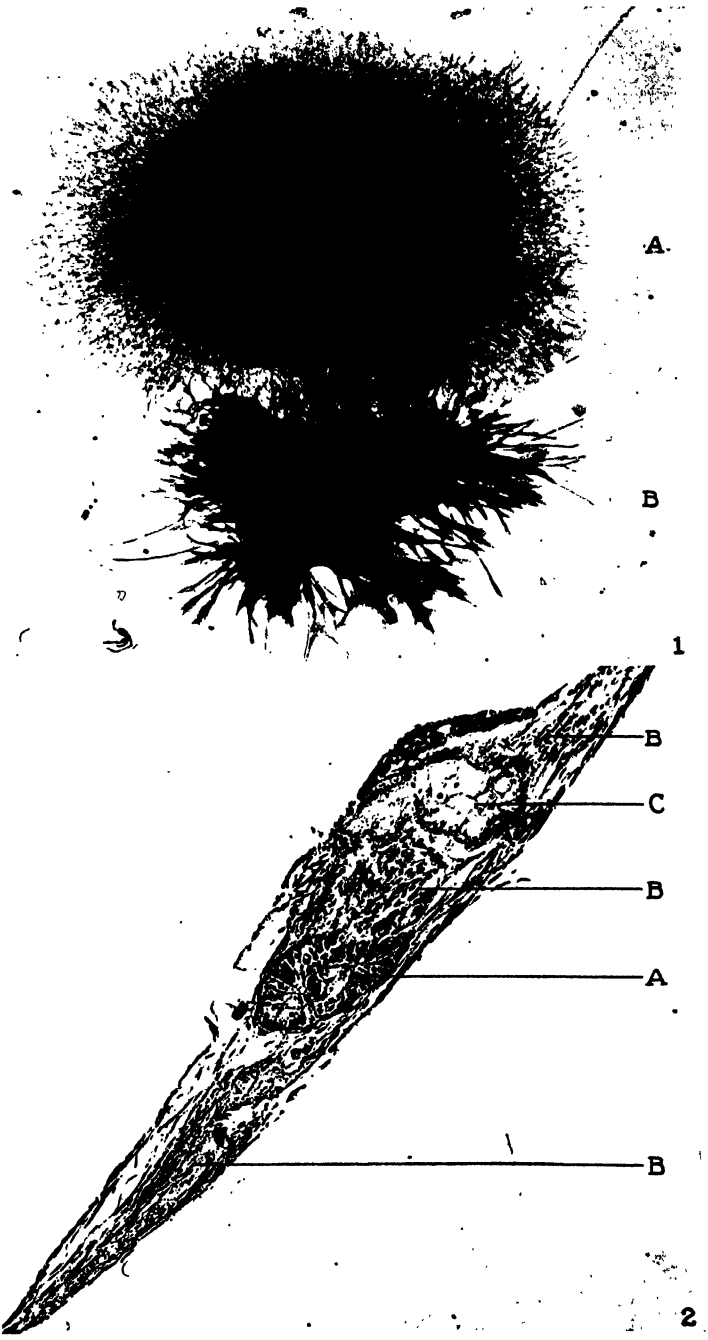
PLATE 17.

FIG. 4. Experiment 25970–2. Seventeenth passage of a pure culture of epithelium growing in a membrane. Fragment cultivated embedded in the clot. Fixed and stained with Azur II after 48 hours incubation. × about 80.

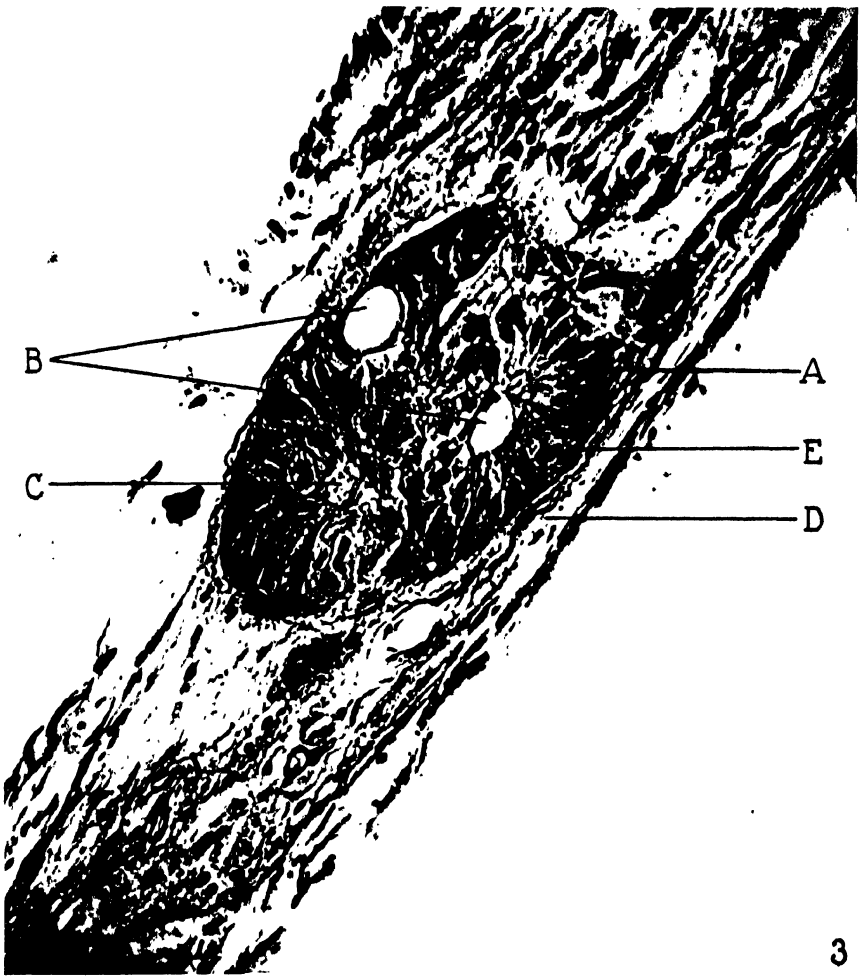
PLATE 18.

FIG. 5. Experiment 25970–2. Same preparation as in Fig. 4. The individual cells appear spindle-shaped and flat. × about 200.

FIG. 6. Experiment 1128–2. Thirteenth passage of a pure culture of epithelium growing embedded in the clot. The new growth appears as solid processes and tubules. Stained with Azur II. × about 160.



(Ebeling and Fischer: Fibroblasts and epithelial cells.)



(Ebeling and Fischer: Fibroblasts and epithelial cells.)

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[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, July 20, 1922, Vol. iv, No. 6, pp. 629–633.]

THE STABILITY OF BACTERIAL SUSPENSIONS.

I. A CONVENIENT CELL FOR MICROSCOPIC CATAPHORESIS EXPERIMENTS.

BY JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 10, 1922.)

Measurements of cataphoresis of particles in an electric field are complicated by the fact that in addition to the motion of the particles relative to the water, the water itself moves at the surface of the cell. In macroscopic measurements the cell is large enough so that the movement of the water as a whole is negligible. If the measurements are made in a narrow cell, however, the motion of the water is very great and must be corrected for. The theory and technique of such measurements have been thoroughly discussed by Ellis[1] and Powis.[2] As Ellis pointed out, the total motion of the water in a closed cell must be zero, since the water which moves one way at the surface of the glass must return in the opposite direction in the center of the cell. The *average* observed motion of the particles at all depths relative to the cell must, therefore, be the true motion of the particles relative to the water, which is the desired value. It is also necessary to use some form of non-polarizable electrodes in order to avoid the formation of gas bubbles. The cell devised by Powis answers the requirements but is troublesome to use if a large number of experiments are made. The cell shown in Fig. 1 has been found very convenient.

Construction of the Cell.—The cell itself is made of a thin slide resting on strips of glass about 0.8 mm. thick cemented to a thick glass slide. Two blocks of thick glass are cemented on top of the cover-slide at each end of the cell. The ends of the cell are then ground smooth on an emery wheel. A piece of thick walled glass tubing is widened and flattened at one end so as to cover the end opening of the

[1] Ellis, R., *Z. physik. Chem.*, 1911, lxxviii, 321; 1912, lxxx, 597.

[2] Powis, F., *Z. physik. Chem.*, 1914, lxxxix, 91.

cell. This flattened end is ground smooth and is cemented to the end of the cell. The same process is repeated at the other end of the cell. The method of arranging the electrodes is apparent from the figure. The best material for cementing the cell together was found to be soft "De Khotinsky cement." The pieces of glass are warmed to about 80° in an air bath, coated with a thin layer of cement and pressed together. The surplus cement is then removed with wire and the last traces are wiped off with a piece of paper moistened with toluene. After the end-pieces have been fastened to the cell it is

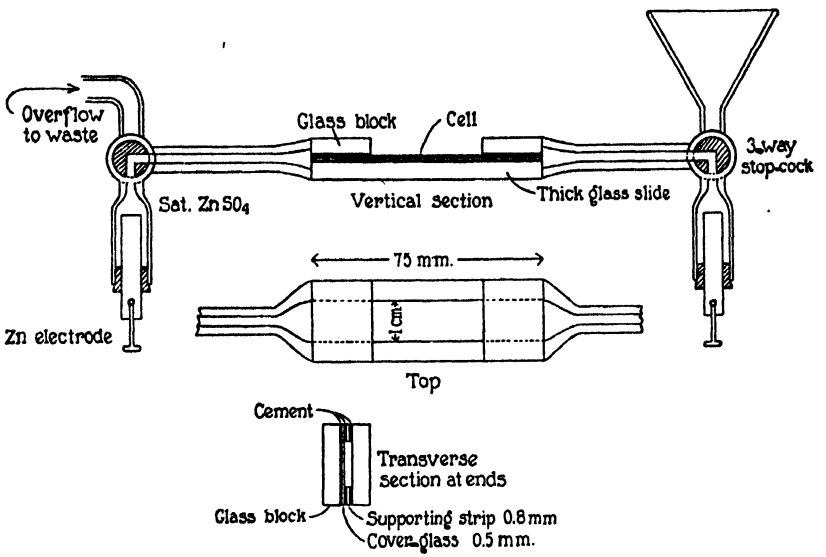

FIG. 1.

advisable to run more cement around the joint to strengthen it; this can easily be done by using a small soldering iron, the cement being handled in the same way as solder.

Calibration of the Cell.—It is necessary to know the drop in potential per cm. in the cell itself. Since the area of the connecting tubes, etc. in general is different from that of the cell it is necessary to correct for this difference. If the apparatus as a whole is filled with the same solution, as is the case during an experiment, the total resistance of the solution will evidently be proportional to the length and inversely proportional to the cross-section. Since the drop in potential per cm.

is proportional to the resistance per cm., it is possible to calculate the drop in potential in the cell itself, provided the dimensions of the rest of the apparatus are known. Expressed as an equation,

$$\text{Drop in potential per cm. in the cell} = \frac{\frac{L_c}{A_c}}{\frac{L_c}{A_c} + \frac{L_t}{A_t} + \frac{L_t'}{A_t'}} \times \text{total drop in potential}$$

L_c = length of cell in cm.
A_c = area of cross-section of cell.
L_t = length of tubing in cm.
A_t = area of cross-section of tubing.
The total potential is measured by a voltmeter connected to the Zn. electrodes.

In experiments with salt concentrations of less than tenth normal the resistance of the zinc sulfate is negligible. In any case the uncertainties due to the change in dielectric constant with increasing salt concentration are probably greater than the error introduced by neglecting the zinc sulfate. It can be seen from the above that failure to allow for a widening of the system at some point would make little or no difference in the potential gradient but that a narrow place in the system even though very short would cause a very large error. It is important, therefore, to be sure that the cell is not narrowed at the ends by the cement or by failure to align the ends of the cell and the side tubes.

Method of Measuring the Velocity of Migration.—The cell is clamped in position under the microscope, after filling the electrode tubes with saturated zinc sulfate, and the electrodes connected to a source of potential. The stop-cocks are turned so as to close the tubes containing the zinc sulfate and the cell filled with the suspension, care being taken to avoid air bubbles. The stop-cocks are then turned so as to connect them with the zinc sulfate solution, the circuit closed and the time required for a particle to cross a division of the micrometer eye-piece determined with a stop-watch. Owing to the migration of the water itself, it is necessary to obtain the average motion of the particles in the cell as a whole. This may be done accurately by determining the speed at various depths, say every 0.05 mm., plotting the curve so obtained and determining the mean height from the area

as measured by a planimeter and the length of the base. This is a time-consuming procedure and Ellis proposed a correction formula which required only two measurements, one at the surface of the cell and one at the center. This formula however, is based on the assumption that the velocity of the water in the center of the cell bears a constant relation to the velocity of the water at the walls of the cell. This ratio in turn depends upon the viscosity of the solution. The writer found that in the presence of proteins, serum, etc., the formula did not hold, owing presumably to changes in viscosity. It was found, however, that a value could be obtained which agreed with the true mean value by making measurements at the middle of each sixth or each eighth of the cell. Since the upper and lower halves of the cell are symmetrical, this requires either three or four measurements. It was found that the results were more reliable if a few measurements were taken at four depths than if the same total number of measurements were made at three depths. The procedure adopted was as follows: The apparent depth (on account of diffraction this is three-fourths of the actual depth) of the cell was 0.64 mm. as measured by the micrometer screw of the fine adjustment. The velocity of the particles was therefore measured at a distance of 0.04, 0.12, 0.20, and 0.28 mm. from the top of the cell, corresponding to the center of the first four eighths of the cell. Four measurements were made at each depth and the average of the reciprocals of these values taken as the true average velocity of the particles relative to the water. It is advisable to have a reversing switch in the circuit and take alternate measurements with the current reversed. These measurements should agree, and any divergence can usually be traced to air bubbles or a leak in the cell. When there is no potential across the cell the particles should remain stationary. Table I is an example of an experiment.

The potential is calculated from the observed velocity by means of the Helmholtz-Lamb equation as discussed in the preceding paper.[3]

Accuracy of the Method.—It was found in general that the measurements could be repeated within 1 to 2 millivolts. The calculated error from one series of measurements is considerably less than that, but the difference is probably due to the difficulty of making the measurements at exactly the correct depth. It is necessary, of course, to count

[3] Northrop, J. H., and Cullen, G. E., *J. Gen. Physiol.*, 1921–22 iv, 635.

only those particles that are sharply in focus. This error could be lessened by using a deeper cell. It was found, however, that in a cell 2 mm. deep there was irregular drifting of the particles. Since the final value depends as a rule on the difference of two experimental values, the percentage error is larger the smaller the velocity.

TABLE I.

Rate of Migration of B. typhosus Suspension in Distilled Water.
Potential gradient = 4.5 volts per cm.

Distance from cover-glass at which determination was made.	Time to go 45 μ.	Average time.	Average μ per second.
mm.	*sec.*	*sec.*	μ
0.04	+15.0 +16.0* +15.5 +14.0	+15.2	+2.95
0.12	−4.5 −5.0 −4.0 −5.0	−4.6	−9.8
0.20	−2.5 −2.3 −2.4 −2.6	−2.45	−18.2
0.28	−2.0 −1.8 −2.0 −2.0	−2.0	−22.5

Average μ per second at all depths..... −11.9
Average μ per second calculated for potential gradient of 1 volt per cm. −2.65

Potential bacteria water, −33.5 millivolts.

* The sign refers to the apparent sign of charge of the particle, *i.e.* + indicates migration to the cathode.

Apparent Reversal of the Charge on the Glass.—It was noted that the direction of migration of the water reversed at times under the same conditions that caused a reversal in the motion of the particles. It is known that the sign of the charge on glass cannot be reversed so that this result is at first sight anomalous. The explanation is simple, however, since microscopic examination of the cell shows that it becomes more or less coated with the organisms, which adhere firmly to the glass. The cell wall is, therefore, no longer glass but is partially composed of the same material as the suspension and therefore reverses its charge under the same conditions as does the suspension.

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AN APPARATUS FOR MACROSCOPIC CATAPHORESIS EXPERIMENTS.

BY JOHN H. NORTHROP AND GLENN E. CULLEN.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 10, 1922.)

The form of apparatus[1] shown in Fig. 1 has been found very convenient for the determination of the migration of fine suspensions in the electric field. Its essential difference from the usual cataphoresis apparatus is that the whole is inverted so that the central portion, which contains the suspension which is to be studied, is above the heavier electrode solutions and may be left open. This increases both the convenience and accuracy of method since (1) a greater latitude in concentration of solution is allowed, (2) the boundaries may be adjusted more exactly, and (3) the solution may be renewed without disturbing the electrode solution. The zinc electrodes are put in place with rubber stoppers, the tube is clamped in a vertical position, and the apparatus is filled with saturated zinc sulfate. The three-way stop-cocks are then closed and the zinc sulfate in the upper part of the cell is washed out through the "tail holes." The tubes above the stop-cocks are now filled with 0.1 M sucrose solution containing the same concentration of electrolyte or other substance as is to be used with the suspension. The sugar solution is then allowed to run out until the level reaches the small tube connecting the funnel and the U-tube. The suspension is then added and the level adjusted carefully by means of the stop-cocks so that the line of demarcation is opposite one of the graduations on the side arms. The upper stop-cock is then closed and the lower ones are opened so as to connect the zinc sulfate with the sugar solutions. The current is applied and the distance traversed by the boundary determined after a convenient interval.

[1] For a discussion of this and similar methods see Burton, E. F., The physical properties of colloidal solutions, London, New York, Bombay, Calcutta, and Madras, 2nd edition, 1921.

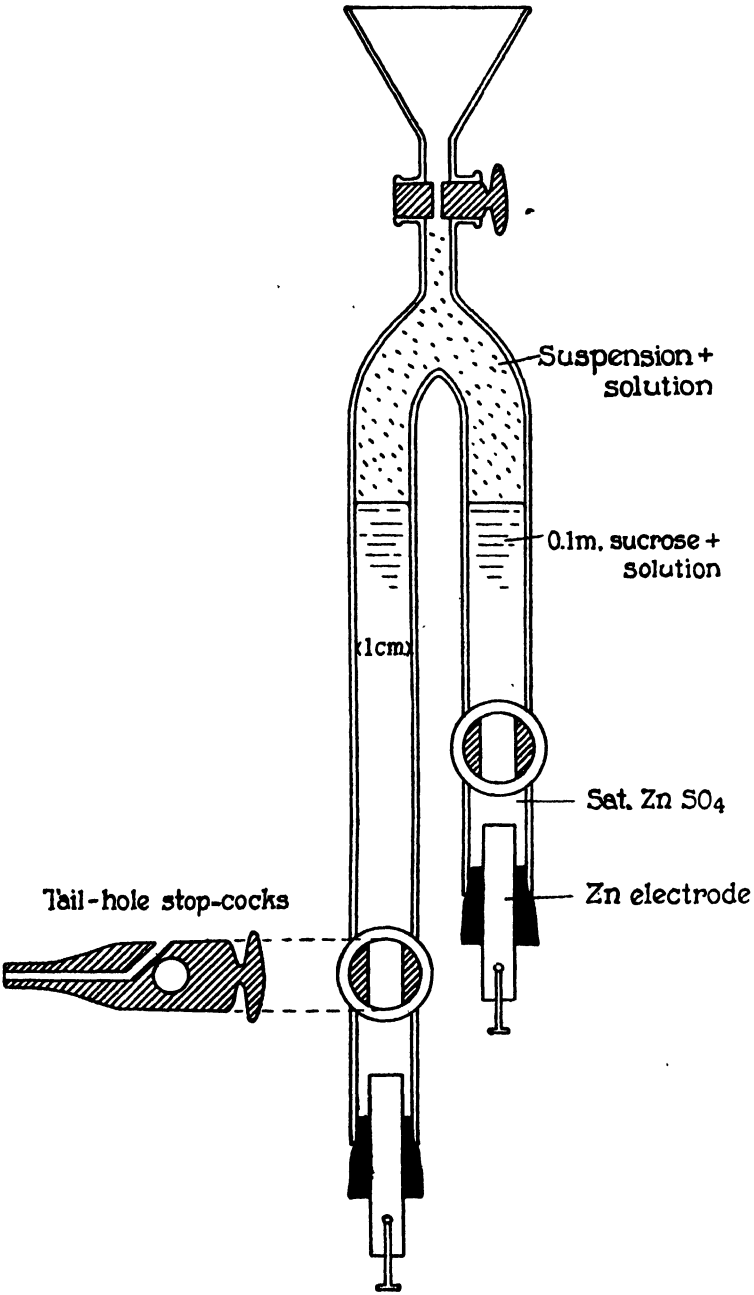
Suspension + solution
0.1m. sucrose + solution
(1cm)
Sat. Zn SO4
Zn electrode
Tail-hole stop-cocks

FIG. 1.

Since the cell is of uniform diameter the drop in potential is determined simply by dividing the total voltage by the distance between the three-way stop-cocks. (In solutions of less than 0.1 N the drop in potential in the saturated zinc sulfate may be considered as negligible; if more concentrated solutions are used it is necessary to apply a small correction for the resistance of the zinc sulfate.)

Influence of the Voltage.—It was found that the rate of migration was directly proportional to the voltage between the limits of 1 to 4 volts per cm., provided the experiment was not allowed to run too long. At low voltages, the rate of migration remained constant until the boundary approached the zinc sulfate, but if the potential drop was increased beyond 2 volts per cm., it was found that the boundary moved at the proper rate for the first 4 to 5 mm., but then became much too slow on one or both sides. There was also a tendency for the boundary to become convex on one side, showing that the migration of the water was interfering with the measurement. If the solution is of high conductivity, the voltage must be still further decreased to prevent heating effects and subsequent convection currents.

Influence of the Sugar Concentration.—The presence of sugar greatly facilitates the adjustment and maintenance of a sharp boundary line. No effect on the velocity of migration could be observed up to 0.5 M. Higher concentrations than this decrease the velocity presumably on account of the viscosity.

Calculation of the Potential from the Velocity of Migration.—The value for the potential difference between the particle and the surrounding solution is calculated by means of the Helmholtz-Lamb equation[1]

$$\text{P.D.} = \frac{4\pi n v}{K X}$$

in which

n = viscosity of the solution.
v = velocity of particle in cm. per second.
K = dielectric constant of the solution.
X = potential gradient; *i.e.*, the drop in potential in E.S.U. per cm.

All electrical units are electrostatic.

Substituting for the viscosity (0.009) and dielectric constant of water at 20° (80), and converting to volts it is found that P.D. in millivolts = 12.6 × μ per sec. × volts per cm. = 4.5 × mm. per hour × volts per cm.

In the experiments to be described no correction was made for a change in the dielectric constant nor viscosity.

The distance is taken as the average of the observed movement in the two arms. This corrects for any gravity effect. As a rule, however, the two readings are nearly identical and any marked discrepancy is apt to be the result of a leak in the apparatus or some other accidental error.

The apparatus has been found to work very satisfactorily for gelatin, edestin, the bacillus of rabbit septicemia, and other fine suspensions. With suspensions containing larger particles such as casein, typhoid bacilli, etc., the line of demarcation becomes blurred and the results are not satisfactory. For any particles that can be seen without an oil immersion lens the microscopic method is better.

Accuracy of the Measurements.—The measurements can usually be repeated as closely as can be read; *i.e.*, ± 0.1 mm. The error is greater in very dilute and very concentrated salt solutions.

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THE STABILITY OF BACTERIAL SUSPENSIONS.

II. THE AGGLUTINATION OF THE BACILLUS OF RABBIT SEPTICEMIA AND OF BACILLUS TYPHOSUS BY ELECTROLYTES.

BY JOHN H. NORTHROP AND PAUL H. DE KRUIF.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 22, 1922.)

It is a very old observation that the stability of suspensions is markedly affected by the addition of electrolytes.[1] Under certain conditions the particles remain separate, while under other conditions they adhere to each other. In the latter case the particles settle rapidly and the suspension is said to be coagulated or agglutinated.[2] Since under certain conditions the particles remain distinct and in others collect into large masses, it is evident that there is a force which tends to hold them together and another force which tends to keep them apart. If the attractive force is greater than the repulsive force, the particles agglutinate. It was early found that nearly all substances in suspension are electrically charged with reference to the surrounding liquid, and it was suggested by Jevons that the repulsion due to this charge was the repelling force. This conception was substantiated by Hardy, who found that suspensions of denatured proteins coagulated at the point at which they carried no electric charge. Hardy called this the isoelectric point. Hardy's experiments have been greatly extended by Michaelis and his coworkers. It is probable, however, that the precipitation of proteins and the agglutina-

[1] For a review of the literature on this subject see Burton, E. F., The physical properties of colloidal solutions, London, New York, Bombay, Calcutta, and Madras, 2nd edition, 1921. In regard to proteins see Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.

[2] The rapid settling of the coagulated suspension is a secondary phenomenon due to the increase in size of the particles and governed presumably by Stokes' law. The primary phenomenon is the adherence or repulsion of the individual particles.

tion of suspensions are governed by entirely distinct forces. In the case of oil emulsions Powis[3] was able to show that agglutination occurred whenever the potential became less than a certain critical value, in this case about 30 millivolts. Powis' experiments leave little doubt that the potential at the oil-water surface is the determining factor in the agglutination of oil emulsions. Burton[1] also found that metallic suspensions coagulate in the zone where the potential is small, although he did not find such a definite critical value.

In the case of bacteria, however, the results have been much less satisfactory. It was found by Bechhold,[4] Arkwright,[5] Teague and Buxton,[6] and others that bacteria were always negatively charged whether or not they were agglutinated. These authors concluded, therefore, that the potential carried by the organisms could not account for the phenomena. Putter[7] was able to show some qualitative agreement between the potential and agglutination of *Bacillus typhosus*.

Results of the Present Experiments.

It is evident that in order to test the hypothesis outlined above, it is necessary to measure both the force which tends to cause the particles to adhere as well as that which keeps them apart, since if both forces are affected by the conditions of the experiment but only one is measured, it will be impossible to interpret the results. The potential may be conveniently measured by the rate of migration in an electric field. The attractive forces, however, have usually been assumed to remain constant and no attempt has been made to measure them. It was found, in the course of the present experiments, that a comparative measure of the attractive forces between the organisms could be obtained by measuring the force required to tear apart two glass plates covered with a film of the bacteria and immersed in the solution which was under investigation. As a result of

[3] Powis, F., *Z. physik. Chem.*, 1914, lxxxix, 186.
[4] Bechhold, H., *Z. physik. Chem.*, 1904, xlviii, 385.
[5] Arkwright, J. A., *J. Hygiene*, 1914, xiv, 261.
[6] Teague, O., and Buxton, B. H., *Z. physik. Chem.*, 1907, lvii, 76.
[7] Putter, E., *Z. Immunitätsforsch., 1 te Abt., Orig.*, 1921, xxxii, 538.

these measurements in conjunction with the measurements of the potential difference, it has been found that whenever the potential difference between the surface of the bacteria and the solution is less than about 15 millivolts the bacteria agglutinate, *provided the cohesive force is not affected.* If the cohesive force is decreased, this critical potential is decreased, and if the cohesive force is made very small, no agglutination occurs even though the potential be reduced to zero. It was further found that all electrolytes tested in concentrations less than 0.01 to 0.1 N affect primarily the potential, while in concentrations greater than 0.1 N the effect is principally on the cohesive force. In the case of bacteria sensitized with immune serum, the cohesive force remains constant and the agglutination can be predicted solely from the measurement of the potential.

Experimental Methods.

Measurement of the Potential.—The potential was determined from the rate of migration as described in the preceding papers.[8] The U-tube method was used for the experiments with the bacillus of rabbit septicemia and the microscopic method with *B. typhosus.*

Measurement of the Cohesive Force.—A piece of thick glass slide was covered with a thin film of very heavy suspension of washed organisms (*B. typhosus*), the film allowed to dry and then heated to 60° for a few minutes. This causes the bacteria to adhere firmly to the glass. A heavy (No. 3) cover-slip was similarly prepared. The cover-slip was suspended by means of a fine platinum wire from the lever of the du Noüy[9] surface tension apparatus. The glass slide was immersed in a dish containing the solution to be studied and the cover-slip allowed to rest on it with its own weight for 1 minute. The force required to pull the cover-slip from the slide was then determined. It was found that if the measurement was made immediately after the two surfaces came in contact, the value obtained depended on the force with which the two had been pressed together. If the slip had been pressed down firmly a much greater force was required than if it had simply been allowed to rest on the slide. After a short time interval, however, this difference became less, and eventually the same reading was obtained in both cases. This is due presumably to the fact that the distance apart of the two surfaces is regulated by capillary forces and comes to the same point from either side. The same smear was used as long as the same value was obtained on replacing the preparation in distilled water. The value obtained becomes less after ten or

[8] Northrop, J. H., and Cullen, G. E., *J. Gen. Physiol.*, 1921–22, iv, 635. Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv 629.

[9] du Noüy, P. L., *J. Gen. Physiol.*, 1918–19, i, 521.

fifteen measurements due to the gradual removal of the film. Control experiments with clean glass surfaces showed no significant variation under the conditions of the experiment. The values obtained in this way were surprisingly reproducible. They have been expressed as milligrams required to separate two surfaces each 2 cm. square. The results are not exactly comparable to the measurements of the potential since the organisms have been subjected to dry heat. It will be noted, in fact, that the results do not conform exactly to those expected from the potential measurements. In the case of NaCl, for instance, the concentration required to affect the cohesive force noticeably, is slightly higher than would be expected from the potential curve.

It has usually been considered that this force is a surface tension effect, but there does not appear to be any conclusive evidence as to its nature. It is better, perhaps, to refer to it simply as "cohesive" without an exact definition of its nature.

Measurement and Regulation of the Hydrogen Ion Concentration.—The pH determinations were made electrometrically, using a saturated calomel cell and taking the pH value of 0.10 N HCl as 1.04 at 33° as the standard.

Buffers Used.—It was found that a very convenient buffer could be made by combining sodium phosphate, sodium acetate, and glycine. It may be used over a range of pH from 1 to 13 and has the further advantage that the nature of ions present is not varied. The only variation is a change in concentration of the Cl and Na ions. The composition and the titration curve of the buffer are given in Fig. 1. This is referred to as G. P. A. Buffer. The pH measurements were made at 33°. In some experiments Walpole's[10] acetate series was used.

Cultures.—The culture of the bacillus of rabbit septicemia used was that previously isolated and described by one of the writers.[11] The typhoid culture was the Pfeiffer strain obtained through the kindness of Dr. Charles Krumwiede to whom we are also indebted for the strong antityphoid horse serum.

Measurement of the Degree of Agglutination.—No satisfactory method could be found for measuring the agglutination quantitatively. Several degrees of agglutination were, therefore, selected and the determinations made on this basis. They were recorded as follows:

\- No agglutination.

\+ Distinct particles visible with a lens, 8 diameters magnification.

\++ Particles visible with the eye alone.

\+++ Suspension almost completely agglutinated and settled but cloudy appearance in the supernatant liquid.

C. Supernatant liquid perfectly clear.

The stage marked C. is the easiest to detect with certainty and was used as the end-point.

The degree of agglutination increases with time at first but after 24 hours remains constant. All readings were therefore made after 24 hours at 20° to elimi-

[10] Walpole, G. S., *J. Chem. Soc.*, 1914, cv, 2501.

[11] De Kruif, P. H., *J. Exp. Med.*, 1921, xxxiii, 773.

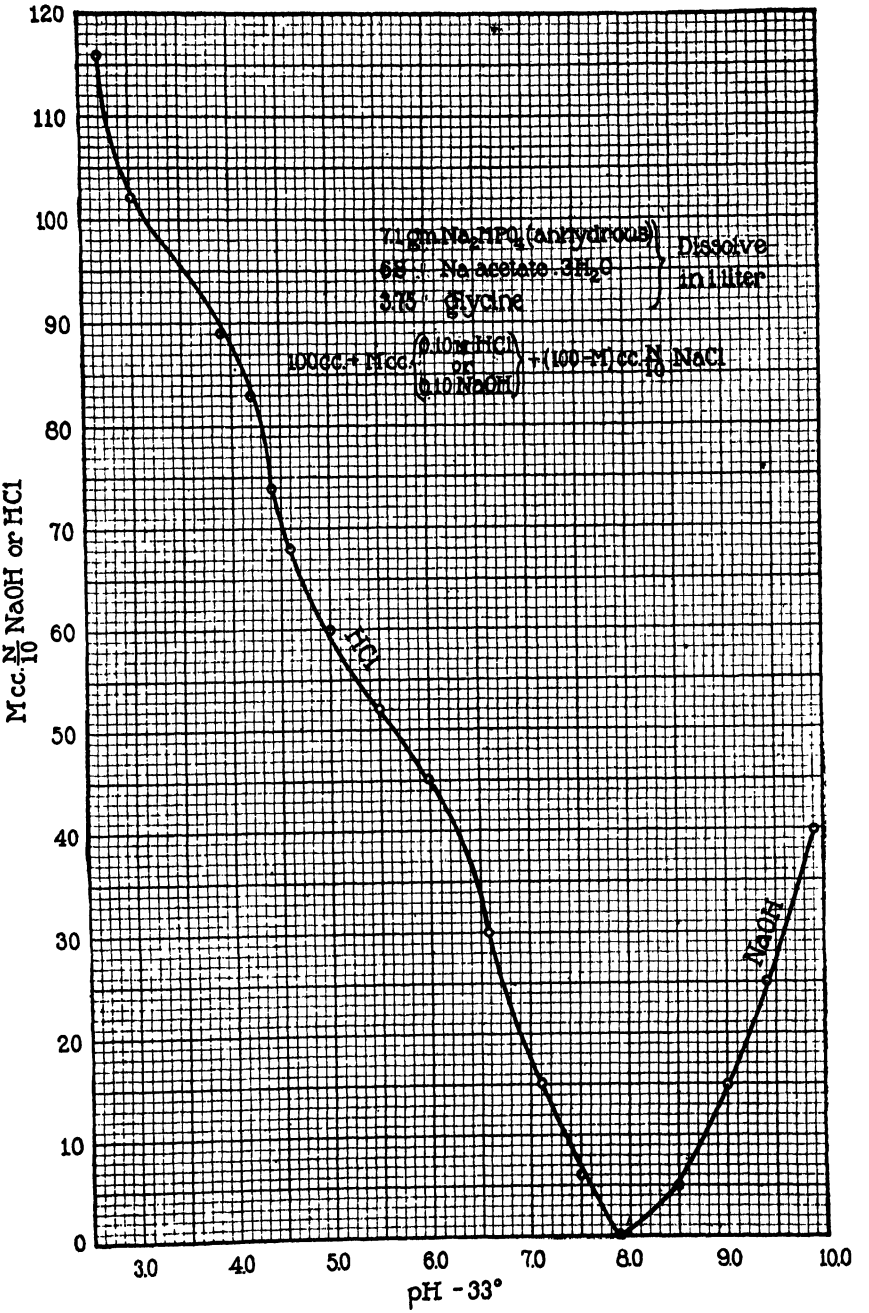

FIG. 1. Titration curve of glycine acetate phosphate buffer.

nate the time factor. A typical experiment is shown in Table I. It is evident that there is some relation between the charge and the rate of agglutination. The suspensions having the lowest charge are the ones which agglutinate the most rapidly. The table shows, however, that the relation is not continuous. Those suspensions having a potential greater than about 15 millivolts do not agglutinate completely at any time. In other words, the potential does not merely effect the time required for agglutination, but if larger than a certain value, prevents it entirely. This is the result obtained by Powis.[3] The fact that the point of agglutination is not sharp but covers a fairly wide range between no agglutination and complete agglutination, may be due to the individual variation in the

TABLE I.

Effect of Potential and Time of Standing on Agglutination.

Suspension of Type D[11] in acetate buffer pH 4.2 + noted concentration of egg albumin pH 4.2.

Concentration of egg albumin.	Mm. per hr.	Potential difference.	Agglutination after time noted at 20°C.				
			0.5 hr.	1 hr.	4 hrs.	24 hrs.	48 hrs.
per cent		*millivolts*					
0	−7.5	−34.0	−	−	−	−	−
0.0003	−6.0	−27.0	−	−	−	+	+
0.001	−4.0	−18.0	−	−	+	++	++
0.003	−2.0	−9.0	−	+	++	C.	C.
0.010	0	0	+	++	C.	C.	C.
0.03	+0.8	+3.4	−	++	++	C.	C.
0.10	2.0	+9.0	−	+	++	C.	C.
0.30	2.5	+11.2	−	−	+	C.	C.
0.90	3.2	+14.4	−	−	−	C.	C.

\+ = agglutination visible with lens (8 diameters).
++ = agglutination visible without lens.
C. = complete settling, supernatant clear.

particles. It would be better theoretically, therefore, to use the point of half coagulation as the end-point. This cannot be determined experimentally owing to the lack of a quantitative method for determining the degree of agglutination.

Preparation of the Suspension.—It has been noted by one of us[12] that the presence of traces of peptones, etc., present in the culture medium, markedly affect the agglutination of the organisms. The suspensions were therefore thoroughly washed in distilled water. 24 hour broth cultures of the organisms were centrifuged, and resuspended in distilled water. This process was repeated four

[12] De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 395. See also Putter.[7]

times. The sediment finally obtained was then suspended in a volume of distilled water equal to one-half that of the original broth. For the determinations one volume of this "standard" suspension was added to one or two volumes of the other solutions used. Table II shows that no noticeable change could be detected after the second washing.

Effect of the Manner of Mixing and Time of Standing on the Potential.— No difference could be detected in the results obtained when the suspension was added to the solution or *vice versa*, provided the mixing was rapid and complete. As a rule the suspension was squirted into the solution from a pipette and mixed as thoroughly and rapidly as possible. No significant changes occurred in the potential measurements over an interval of 2 days except in the case of silver salts. The effect on the potential is, therefore, almost instantaneous in most cases. This is also true of the effect of immune serum, and shows that the time element consists in the time required for the organisms to come into contact.[18] In

TABLE II.

Effect of Washing on Rate of Migration of Type D Suspension.

100 cc. of broth culture, Type D, were centrifuged, suspended in distilled H_2O, centrifuged, and the process was repeated as noted. Migration was determined as noted.

No. of times washed	0	1	2	3	4
Potential at pH 4.4	−9.0	−18.0	−27.0	−28.0	−28.0
" " pH 3.0	−13.0	+1.6	+1.6		+1.8

the case of suspensions treated with silver salts at a pH of 4 or more, the potential drops rapidly and is very much lower after 24 hours. At the same time the suspension turns black so that the effect is probably due to the reduction of the silver.

EXPERIMENTAL RESULTS.

The results of the experiments are shown graphically in Figs. 2 to 8. The calculated potential in millivolts between the surface of the organism and the surrounding liquid is plotted as ordinates, and the salt concentration as abscissae. Since there is some doubt as to the correctness of the formula connecting velocities to millivolts, the actual velocities corrected for a potential drop of 1 volt per centimeter have also been given. The degree of agglutination is indicated by the character of the line. In the experiments in which no pH value

[18] This conclusion had been reached by F. L. Gates (*J. Exp. Med.*, 1922, xxxv, 63) in a study of the time required for adsorption of immune body.

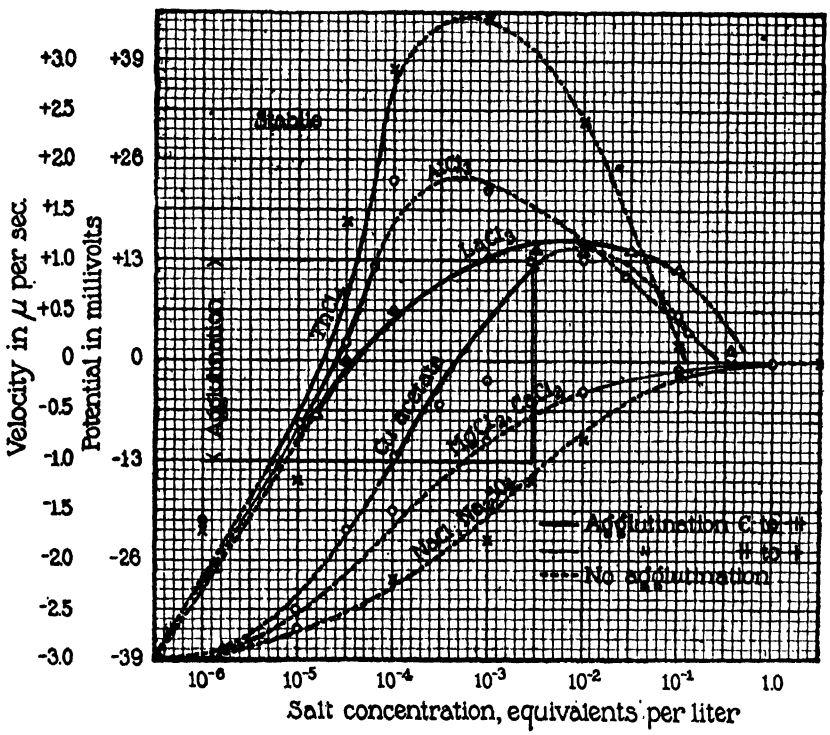

FIG. 2. Effect of salts on the potential and agglutination of *B. typhosus*.

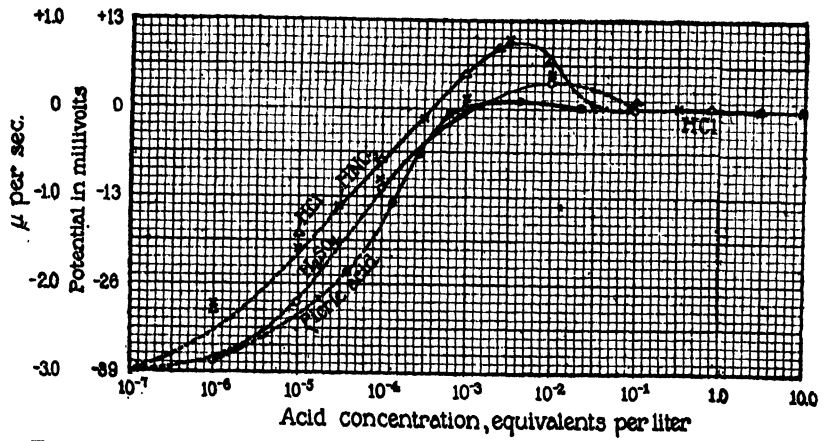

FIG. 3. Effect of acids on the potential and agglutination of *B. typhosus*.

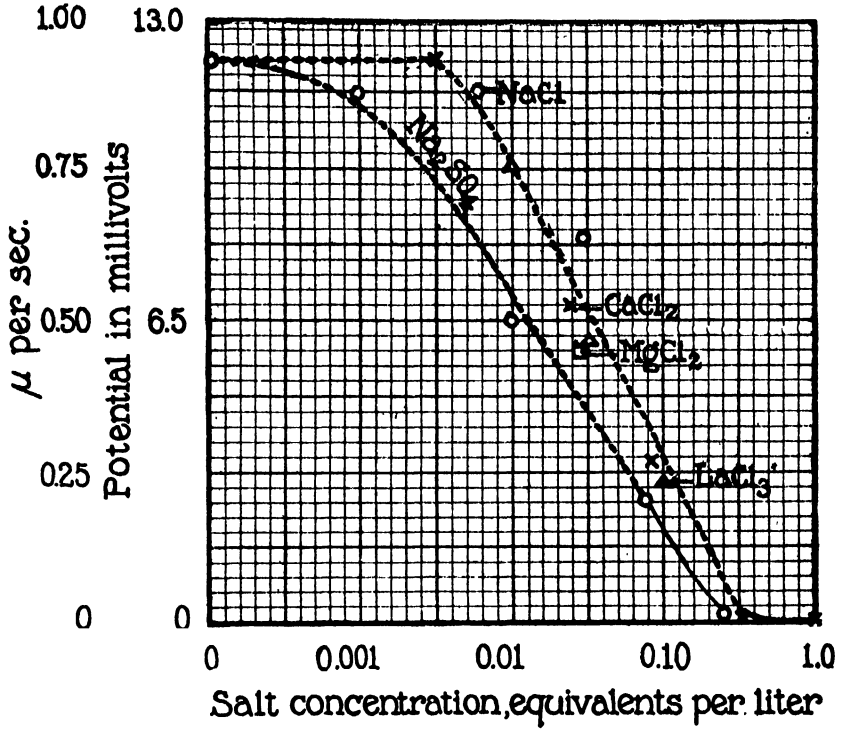

FIG. 4. Effect of salts on the potential and agglutination of *B. typhosus* at pH 2 (0.01 N HCl).

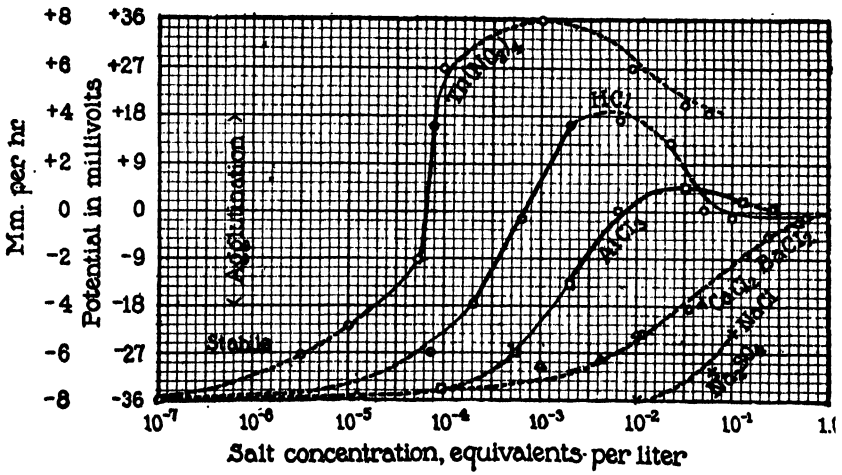

FIG. 5. Effect of salts on the potential and agglutination of the bacillus of rabbit septicemia Type D strain.

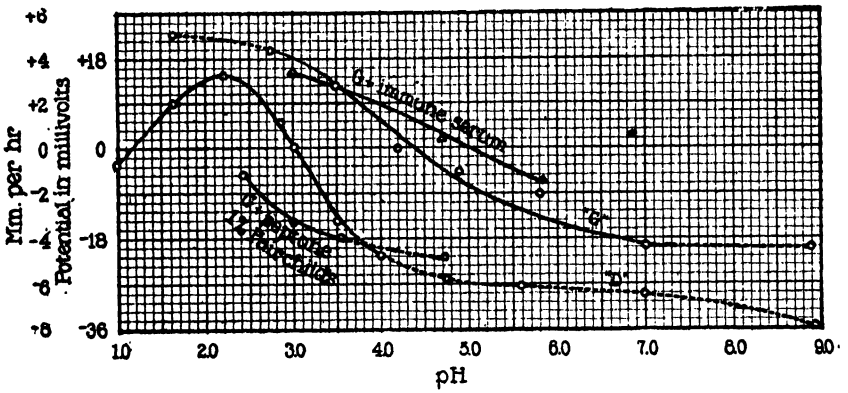

FIG. 6. Comparison of the acid agglutination of Type D and Type G strains of the bacillus of rabbit septicemia and the effect of immune serum and peptone on the potential and agglutination of Type G.

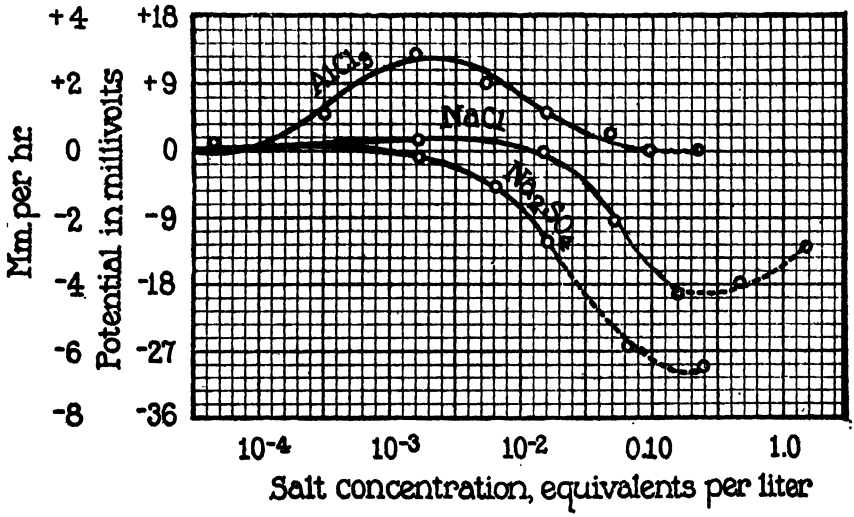

FIG. 7. Effect of salts on the potential and agglutination of Type D at pH 3 (0.001 N HCl).

is given, the pH was not regulated and the results are due in part to changes in the hydrogen ion concentration.

Inspection of the charts shows that in all experiments there is complete agglutination as soon as the potential is reduced below a value of about 15 millivolts (either positive or negative) provided the salt concentration is below 0.001 N. Below this salt concentration, therefore, the agglutination is seen to depend solely on the potential. Any substance which reduces the potential below about 15 millivolts will cause agglutination. There is another range of salt concentration above 0.10 N in which no agglutination occurs, although there is no measurable potential. Between these two ranges of salt con-

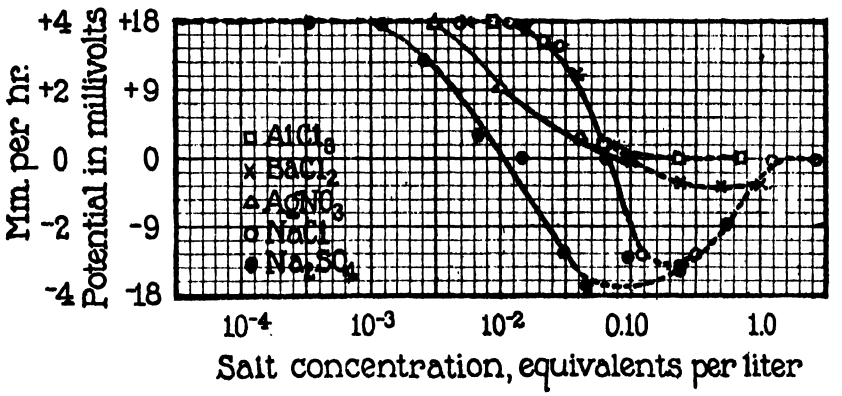

FIG. 8. Effect of salts on the potential and agglutination of Type D at pH 2.0.

centration there is a zone in which agglutination occurs at various potential levels. This is evidently the result that we would expect if the salt acted in low concentration primarily on the potential, and in high concentration on the cohesive force. There would be an intermediate zone in which the agglutination could not be predicted from either measurement alone. This explanation is borne out by the measurements of the cohesive force shown in Fig. 9. These show that the cohesive force is markedly decreased in concentrations of more than 0.01 N; *i.e.*, the range in which the critical potential begins to decrease. The figure shows that the effect on the cohesion is not connected with the valency nor with the electrical effects of the ions. $LaCl_3$ is far more effective than NaCl in reducing the potential, but

less effective in reducing the cohesive force. The agglutination depends on both factors. It is possible, therefore, for all monovalent ions to affect the potential in the same way but to differ in their coagulating power. In order to predict the coagulating efficiency of a salt, it is therefore necessary to know the effect on both the potential and cohesion.

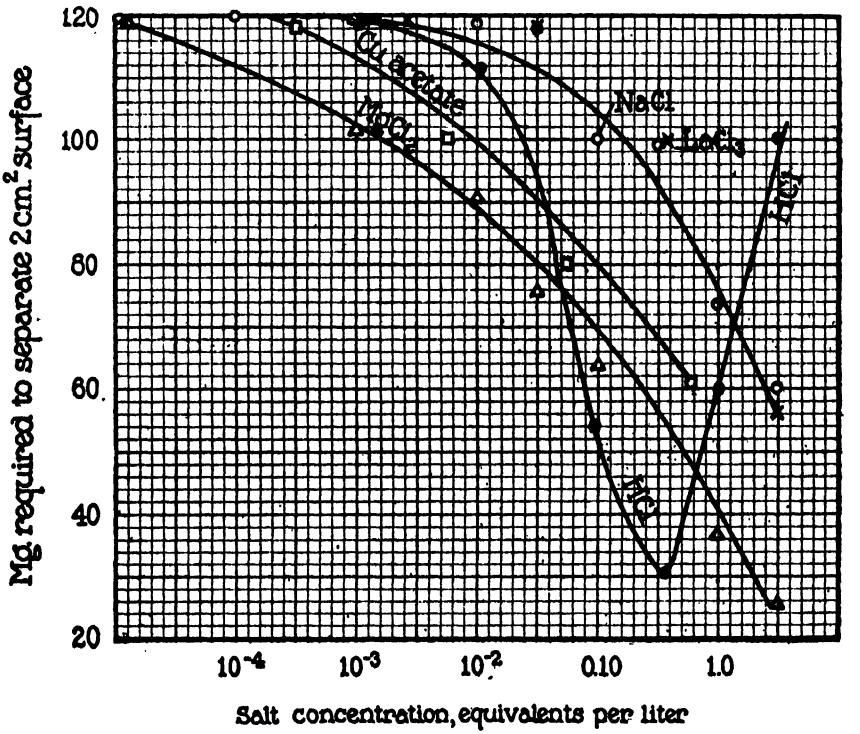

FIG. 9. Effect of salts and acids on the cohesive force between films of *B. typhosus*.

The HCl curve differs from the others in that the cohesion is *increased* in solutions of higher concentration than 0.3 N. This agrees with the agglutination test (Fig. 3) which shows a zone of agglutination at this concentration.

The experiments show the result usually obtained in such cases, that low concentrations of salt precipitate and higher concentrations stabilize again. They also show that this is due in most cases to the

fact that excess salt or acid confers a high potential upon the particles, of opposite sign to that in low concentration.

These various effects are all shown in the case of thorium chloride (Fig. 2). In concentration below 5×10^{-6} N no agglutination takes place since the potential is greater than 15 millivolts (the organisms being negative to the water) and the organisms are kept apart by the repulsion due to this potential. In concentrations between 5×10^{-6} and 5×10^{-5} N there is agglutination, since in this range the potential is less than 15 millivolts and the repulsion is therefore not sufficient to overcome the cohesion. In concentrations of from 5×10^{-5} to 5×10^{-1} the potential is greater than 15 millivolts (though of the opposite sign) and the suspension is again stable. At a concentration above 0.05 N the potential drops below 15 millivolts but agglutination does not occur since the cohesive force has also been reduced. A smaller potential is therefore sufficient to prevent agglutination. At a concentration of 0.10 N the potential is reduced practically to zero and agglutination again occurs. In still higher concentration the organisms are again stable due to a further decrease in the cohesive force.[14] The hydrochloric acid curve is interesting in that it shows a zone of agglutination in concentrated solutions (> 0.3 N). This is due to the sudden increase in the cohesive force at this point as is shown in Fig. 9. This does not occur with the other chlorides and in the latter solutions no agglutination occurs in this range.

The stabilizing effect of sodium chloride in high concentration is shown more strikingly in Fig. 10,[15] which gives the result of adding increasing salt on the acid agglutination zone. The addition of

[14] According to O. Porges (*Centr. Bakt., 1 te Abt., Orig.*, 1906, xl, 133) agglutination occurs again in very strong salt solutions such as half saturated $(NH_4)_2SO_4$. This is probably a salting out phenomenon, due to a decrease in the forces between the surface of the particle and the liquid. For a review of the effect of salts on agglutination see Buchanan, R. E., *J. Bact.*, 1919, iv, 82. The experiment itself shows that this is a different phenomenon since in saturated $(NH_4)_2SO_4$ agglutination occurs immediately whereas the type of agglutination studied in this paper requires considerable time.

[15] It will be noted that in this experiment the isoelectric point was about pH 4.2 while in others with *B. typhosus* (Fig. 3) it is about 3.5. This difference was noted several times and depends probably on the age and condition of the suspension.

0.01 N salt decreases the potential and broadens the agglutination zone slightly. More concentrated salts, however, although it reduces the potential still more, *decreases* the agglutination, since the cohesive force is now being reduced. In concentrations of more than 1.0 N no agglutination occurs. The salt also shifts the zone of agglutination to the acid side. This result has been obtained by Michaelis and Rona[16] with proteins.

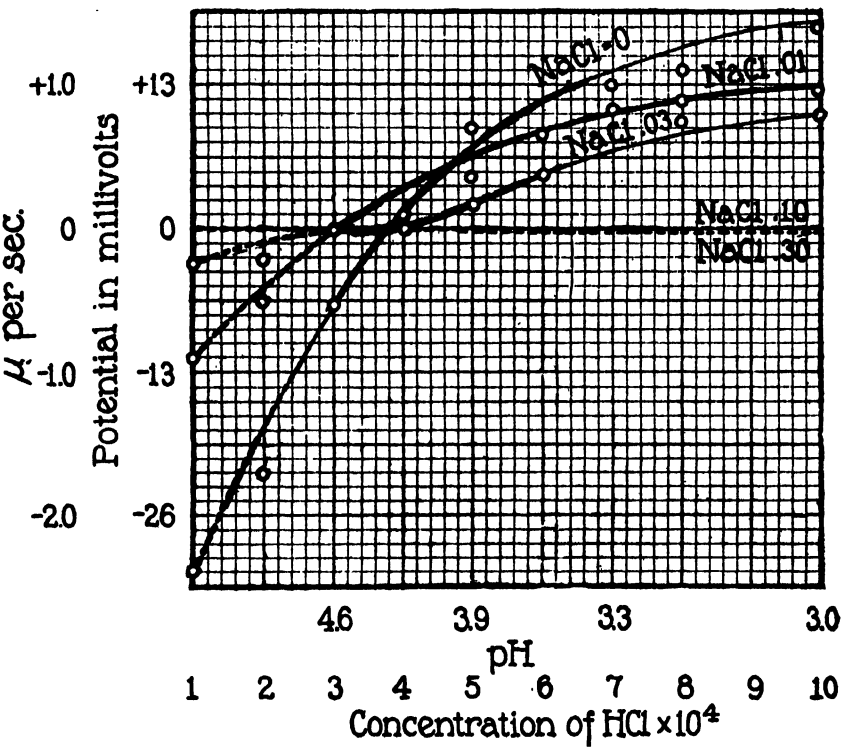

FIG. 10. Effect of NaCl concentration on the potential and agglutination of *B. typhosus* at the acid agglutination zone.

Effect of the Salts on the Potential.—The experiments show the familiar result that the effect is due to the oppositely charged ion and increases in general with the valence of the ion. The effect is not purely due to the valence since the hydrogen ion is far more active than the other monovalent ions. The result also depends on the nature of the

[16] Michaelis, L., and Rona, P., *Biochem. Z.*, 1919, xciv, 225.

suspension since the charge on the bacillus of rabbit septicemia may be reversed by sulfate or NaCl while with *Bacillus typhosus* suspension the charge is reduced but does not change in sign.

The experiments in Fig. 6 show clearly the reason for the characteristic difference in the stability of Types D and G of the rabbit septicemia bacillus.[11] Type D which is very stable has a high potential whereas the potential of Type G is very little more than the critical.

The same figure shows that the acid agglutination zone may be shifted markedly by the addition of other substances. Peptone for instance moves it far to the acid side (*cf.* Putter[7]) while immune body brings the isoelectric point to nearly 5. This point will be discussed more fully in the succeeding paper.

Origin of the Potential.—Loeb has shown,[17] in the case of a protein solution separated from a solution of electrolyte by a collodion membrane, that the charge on the protein solution can be quantitatively accounted for on the basis of Donnan's theory of membrane potentials. According to this theory, electrolytes affect the potential of a particle in two ways. (1) By combining chemically with the particle (for example hydrogen ions). The ion then becomes part of the molecule of which the particle (membrane) is composed. As a result the concentration of this ion differs on the opposite sides of the membrane and gives rise to a potential. This potential may be calculated by Nernst's formula from the concentration of the common ion on both sides of the membrane. The membrane behaves as a reversible electrode for this ion. (2) Ions which affect the distribution of the common ion without further chemical combination with the membrane. This mechanism will suffice to account for all the observations made in the course of this work, if it be supposed that other ions than the hydrogen ion may act by chemical combination.[18] The experiments are more complicated than those with a collodion membrane since the organisms are apparently more or less impermeable to ions.[19]

[17] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667; 1921–22, iv, 351; Proteins and the theory of colloidal behavior, New York and London, 1922, 120.

[18] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 164, 165; *J. Gen. Physiol.*, 1921–22, iv, 463; also two papers in this number of the *Journal* which the writer has had the privilege of reading in manuscript form (*J. Gen. Physiol.*, 1921–22, iv, 741, 759).

[19] Shearer, C., *Proc. Cambridge Phil. Soc.*, 1916–19, xix, 263.

SUMMARY.

1. Measurements have been made of the potential and of the cohesive force at the surface of *Bacillus typhosus* and the bacillus of rabbit septicemia in solutions of various salts and acids.

2. Electrolytes in low concentration (0.01 N) affect primarily the potential, and in high concentration decrease the cohesive force.

3. As long as the cohesive force is not affected, agglutination occurs whenever the potential is reduced below about 15 millivolts.

4. When the cohesive force is decreased the critical potential is also decreased, and in concentrated salt solution no agglutination occurs even though there is no measurable potential.

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THE STABILITY OF BACTERIAL SUSPENSIONS.

III. AGGLUTINATION IN THE PRESENCE OF PROTEINS, NORMAL SERUM, AND IMMUNE SERUM.

BY JOHN H. NORTHROP AND PAUL H. DE KRUIF.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 24, 1922.)

It has frequently been noted that the addition of a small amount of certain substances, especially proteins, markedly affects the behavior of suspensions. It was found by Whitney and Blake[1] for instance that the sign of the charge of gold particles in the presence of gelatin could be reversed by acids, a result which did not occur without gelatin. The same effect has been noted by Loeb[2] in the case of collodion membranes treated with different proteins. The membrane always acquires the isoelectric point of the protein used. It was found by one of the writers that peptone markedly affects the acid agglutination of the bacillus of rabbit septicemia. It has been shown in the preceding paper[3] that the isoelectric point was also displaced. This result had been noted by Putter.[4] The present paper contains the results of experiments on the effect of proteins and sera on the properties of suspensions of bacteria.

Fig. 1 shows the effect of various concentrations of egg albumin on the agglutination and charge of the bacillus of rabbit septicemia (Type D strain). The method of plotting is the same as in the preceding paper. Increasing the amount of egg albumin gradually shifts the curve to the alkaline side so that the isoelectric point is moved to pH 5.0 which is approximately that of egg albumin. In other

[1] Whitney, W. R., and Blake, J. C., *J. Am. Chem. Soc.*, 1904, xxvi, 1339.

[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 659; 1921–22, iv, 213.

[3] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639.

[4] Putter, E., *Z. Immunitätsforsch., Orig.*, 1921, xxxii, 538. The same observation had been made independently by one of the writers De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 345.

words, the particles act more and more like particles of egg albumin. The form of the curve is very similar to the curve found by Loeb[5] for the potential between a solution of egg albumin in a collodion sac and the surrounding solution. As was found in the experiments described in the preceding paper, agglutination occurs whenever the potential is reduced below a value of about 15 millivolts. The result of the addition of egg albumin is, therefore, that the agglutination zone is moved to the alkaline side and that at a pH of 3 the egg albumin stabilizes the suspension instead of precipitating it.

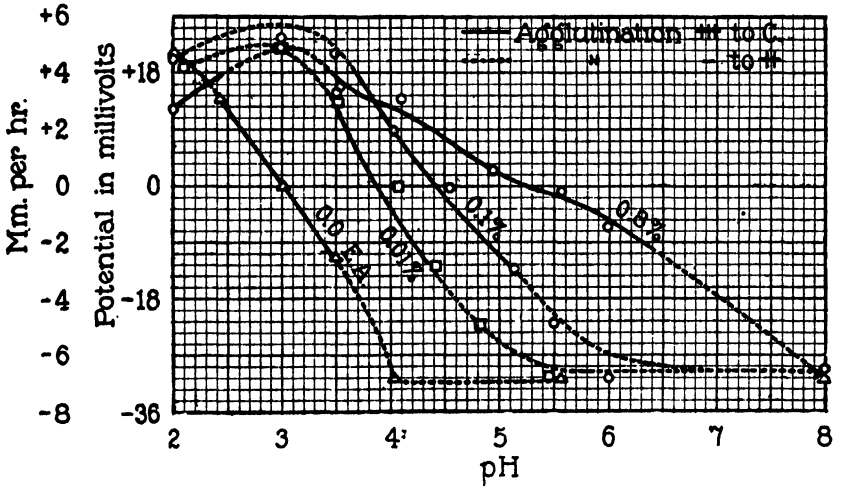

FIG. 1. Effect of the concentration of egg albumin at different pH on the potential and agglutination of Type D. pH adjusted with acetate buffers.

This is typical of the action of protective colloids and is due, as the figure shows, to the increase in the potential. The figure also shows that the amount of egg albumin required to agglutinate is a minimum near the isoelectric point of the suspension and increases as the pH is moved to the alkaline side. Similar experiments have been published by Eggerth and Bellows.[6]

Fig. 2 shows the effect of the addition of globin to a suspension of Type D; the isoelectric point is now shifted to pH 6.5 which is near

[5] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

[6] Eggerth, A. H., and Bellows, M., *J. Gen. Physiol.*, 1921–22, iv, 669.

the isoelectric point of globin. Agglutination again occurs whenever the potential is less than 15 millivolts.

The effect of normal and immune serum on the pH curves of *Bacillus typhosus* is shown in Fig. 3.[7] The result is very similar to egg albumin. It will be noted that there is no marked difference between the immune and the normal serum and also that the isoelectric point is shifted to a pH of 4.7 in both cases. This was an unexpected result, since the isoelectric point of blood globulin is given by Mi-

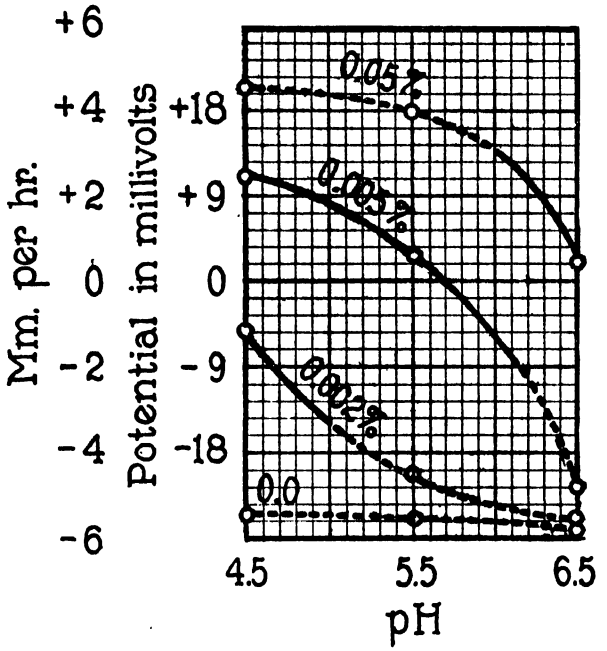

FIG. 2. Effect of the concentration of globin on the potential and agglutination of Type D at different pH. Acetate buffers.

chaelis[8] as 5.4 and the antibodies are known to be associated with the globulins. According to the present experiment, however, the substance in the serum which has the greatest effect on the charge of the

[7] Similar experiments have been made by Kōsaka and M. Seki, Communication to the Okayama Medical Society, *Okayama-Igakkwai Zasshi*, 1922, No. 386.

[8] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914. It is doubtful if this can be considered the isoelectric point of pure immune body since traces of foreign proteins have such a marked effect.

organisms has an isoelectric point at about pH 4.7. The small difference in the concentration of normal and immune serum required to change the isoelectric point renders it improbable that this effect can be ascribed to the immune body.

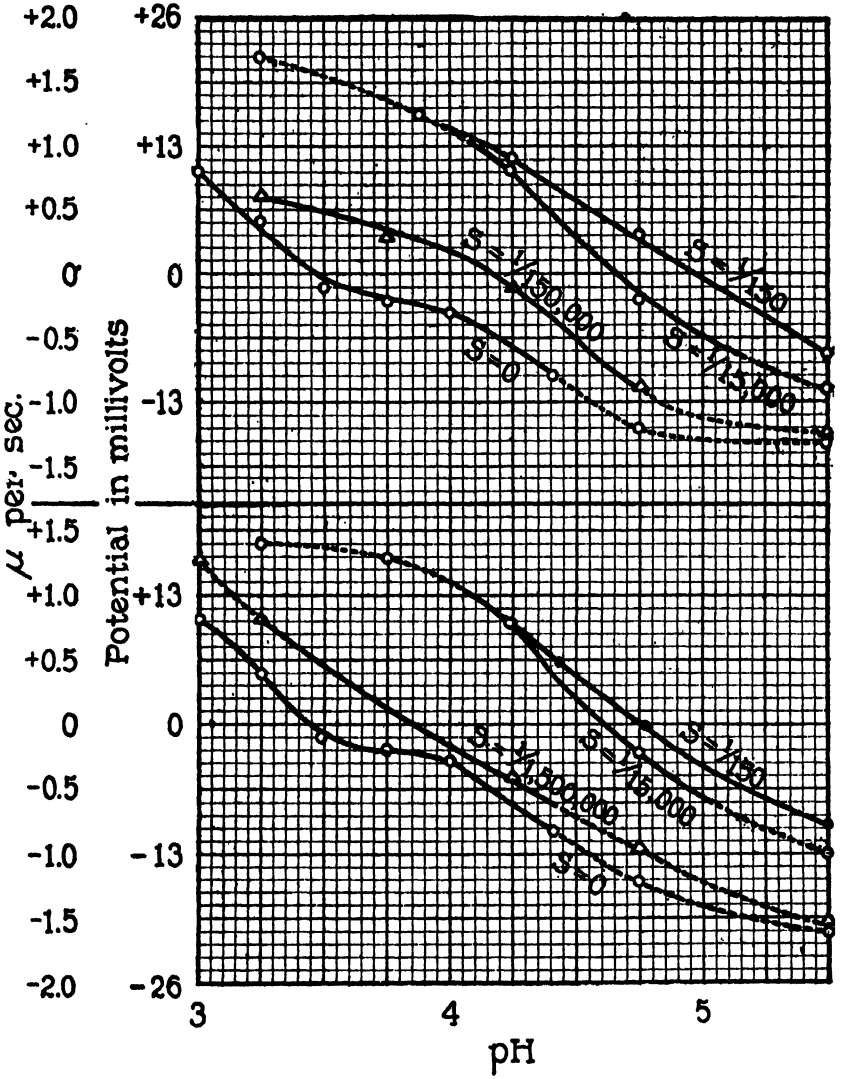

FIG. 3. Effect of different concentrations of normal (upper half) and immune serum (lower half) on the potential and agglutination of *B. typhosus* at different pH 0.01 N acetate buffer.

The stabilizing effect of the serum at pH 3 is likewise due to some constitutent of the serum other than the immune body as is shown by the following experiment. A suspension of *Bacillus typhosus* was treated with an excess of immune serum in 0.10 N salt and then washed once with distilled water. The suspension was then added to

TABLE I.

Agglutination of B. typhosus by Antityphoid Horse Serum at Various C_H. G. P. A. Buffer.

pH	Concentration of serum. 5×10^{-4}	2.5×10^{-4}	1.25×10^{-4}	6.2×10^{-5}	3.1×10^{-5}	1.55×10^{-5}	7.8×10^{-6}	3.9×10^{-6}	Control. No serum.
8.5	C.	C.	C.	C.	C.	++	−	−	−
7.5	C.	C.	C.	C.	C.	++	+	−	−
6.0	C.	C.	C.	C.	C.	++	+	−	−
5.5	C.	C.	C.	C.	C.	++	+	−	−
5.2	C.	C.	C.	C.	C.	C.	+	Tr.	−
5.0	C.	C.	C.	C.	C.	C.	++	+	−
4.6	C.	C.	C.	C.	C.	C.	C.	C.	Tr. +
3.9	C.	C.	C.	C.	C.	C.	C.	C.	C.
3.3	+	+	+	++	++	C.	C.	C.	C.
2.7	+	+	+	+	+	+	+	+	+

Normal Horse Serum.

	pH	2×10^{-3}	1×10^{-3}	5×10^{-4}	2.5×10^{-4}	1.25×10^{-4}	0.6×10^{-4}
Normal horse serum 1.0 cc. + typhoid suspensions 1.0 cc.	5.0	Tr.	Tr.	−	−	−	−
	4.7	+	Tr.	Tr.	−	−	−
	4.4	+	+	Tr.	+	++	++
	3.9	−	Tr.	+	C.	C.	C.
	3.3	−	−	+	+	++	++

pH 3 buffer. The result was instantaneous and intense agglutination, whereas Fig. 3 shows that in the presence of excess serum no agglutination occurs at a pH of 3.

Tables I to IV show the effect of the pH on the amount of normal serum and immune serum to cause agglutination of various organisms.

TABLE II.

Agglutination of Types I and II Pneumococci by Pneumococcus Type I Antiserum at Various C_H. G. P. A. Buffer.

	pH	Dilution of serum. 1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Control. No serum.
Pneumococcus Type I serum dilutions 1.0 cc. + pneumococcus Type I suspensions 1.0 cc.	8.5	C.	C.	C.	++	Tr.	−	−	−	−	−
	7.1	C.	C.	C.	++	+	−	−	−	−	−
	6.0	C.	C.	C.	++	+	−	−	−	−	−
	5.5	C.	C.	C.	++	+	−	−	−	−	−
	5.2	C.	C.	C.	C.	+	+	+	Tr.	Tr.	−
	5.0	C.	C.	C.	C.	++	+	+	+	Tr.	−
	4.6	++	++	C.	C.	C.	C.	++	+	Tr.	Tr.
	3.9	+	+	+	+	+	++	C.	C.	C.	C.
Pneumococcus Type I serum dilutions 1.0 cc. + pneumococcus Type II suspensions 1.0 cc.	8.5	+	+	−	−	−	−	−	−	−	−
	7.1	+	+	Tr.	−	−	−	−	−	−	−
	6.0	++	++	+	Tr.	−	−	−	−	−	−
	5.5	++	++	+	Tr.	−	−	−	−	−	−
	5.2	++	++	+	Tr.	Tr.	−	−	−	−	−
	5.0	++	++	+	+	Tr.	Tr.	−	−	−	−
	4.6	C.	C.	C.	++	+	+	Tr.	Tr.	Tr.	−
	3.9	+	+	C.	C.	C.	C.	C.	++	+	+

TABLE III.

Agglutination of Type G Rabbit Septicemia Bacillus by Rabbit >G at Various C_H.

pH	Dilution of serum. 1:10*	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	Control. No serum.
7.9	++	++	+	+	−	−	−	−	−	−	−	−
7.5	++	C.†	++	+	Tr.	−	−	−	−	−	−	−
7.0	++	C.	C.	++	++	+	Tr.	−	−	−	−	−
6.5	C.	C.	C.	C.	++	+	+	Tr.	Tr.	Tr.	Tr.	Tr.
6.0	C.	C.	C.	C.	C.	++	+	+	Tr.	Tr.	Tr.	Tr.
5.5	C.	C.	C.	C.	C.	C.	++	++	+	+	+	+
5.0	C.	C.	C.	C.	C.	C.	C.	++	++	++	C.	C.
4.5	+	+	+	C.	C.	C.	C.	C.	C.	C.	C.	C.
2.2	−	−	−	−	−	−	−	−	−	−	−	−

* Represent *serum dilutions*. Divide by 2 to obtain final dilution.

† C = Complete agglutination.

++ = Strong agglutination with sediment but turbid supernatant.

\+ = Slight agglutination.

Tr. = Trace.

TABLE IV.

pH	Dilution of normal serum.										Control. No serum.
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	
7.0	+	+	−	−	−	−	−	−	−	−	−
6.0	+	+	+	+	+	+	Tr.	Tr.	Tr.	Tr.	Tr.
5.5	+	+	+	+	++	++	++	++	++	++	++
5.0	+	++	++	++	++	C.	C.	C.	C.	C.	C.
4.5	++	++	++	++	++	C.	C.	C.	C.	C.	C.
4.0	+	+	++	++	++	C.	C.	C.	C.	C.	C.
3.0	+	+	+	+	+	++	++	++	++	++	C.
2.0	−	−	−	−	−	−	Tr.	Tr.	Tr.	Tr.	Tr.

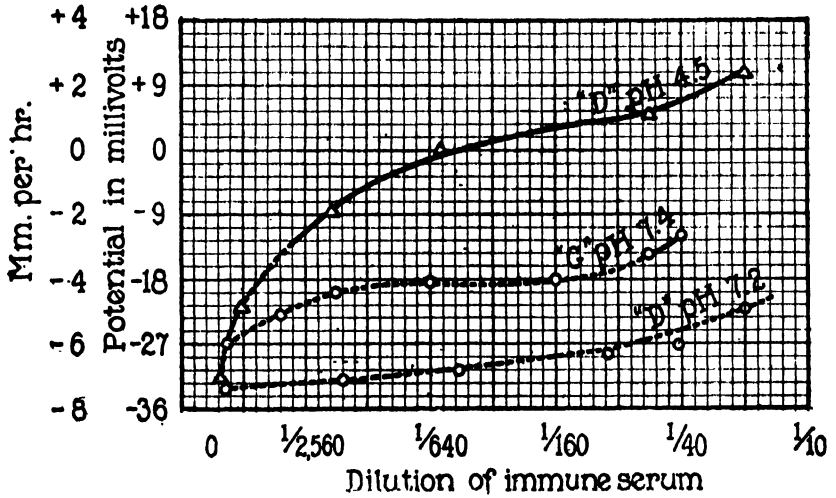

FIG. 4. Effect of immune serum on the potential and agglutination of Type D and Type G at different pH. G. P. A. Buffer.

The results are the same as with egg albumin. In every case the acid agglutination zone is shifted to the alkaline side, and the amount of serum required to agglutinate is a minimum near the isoelectric point of the organism. This result had been noted by Krumwiede and Pratt,[9] and by Michaelis and Davidsohn.[10] It is noticeable also that

[9] Krumwiede, C. K., Jr., and Pratt, J., *Z. Immunitätsforsch., Orig.*, 1913, xvi, 517.

[10] Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1912, xlvii, 59.

the difference between the normal and the immune serum becomes less and less marked as the pH approaches that of the acid agglutination zone of the organism.

Fig. 4 shows the effect of immune serum on the charge and agglutination of Types D and G. As in all the experiments, the agglutination becomes complete as soon as the charge is reduced below 15 millivolts. The figure shows that Type D is difficult to agglutinate because it has a fairly high charge at a pH of 7.2 and the effect of the immune serum is insufficient to reduce this to the critical value. Type G, however, has a lower charge and is much more readily agglutinated. Type D at a pH of 4.5 is easily agglutinated since at this pH the serum has a much greater effect on the charge.

The Effect of Salts.

Bordet[11] showed that salt greatly increased agglutination with immune serum. Porges,[12] however, found that with very powerful immune serum agglutination occurred even though the serum was dialyzed and no salt was present.

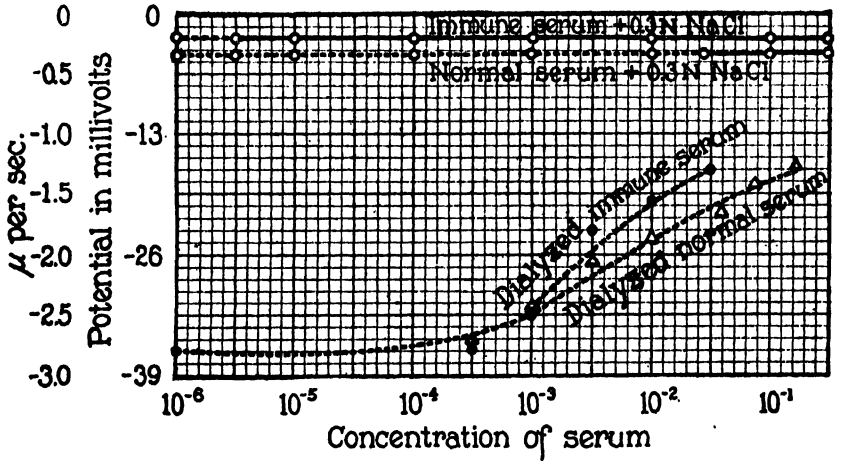

FIG. 5. Effect of dialyzed normal and immune serum on the potential and agglutination of *B. typhosus*. Upper curves show the effect in the presence of 0.3 N NaCl. The potential in these two curves is identical but they have been separated slightly in the figure in order to show the difference in the agglutination.

[11] Bordet, J., Traité de l'immunité dans les maladies infectieuses, Paris, 1920.

[12] Porges, O., *Centr. Bakt., 1 te Abt., Orig.*, 1905, xl, 133.

The effect of dialyzed normal and of powerful antityphoid horse serum on the potential and agglutination of *Bacillus typhosus* is given in Fig. 5. The two upper curves are the results in 0.3 N NaCl. There was no complete agglutination in the absence of salt and no marked difference between the normal and immune serum, although both affect the potential. (The serum was prepared by dialysis against distilled water and then dissolved by the addition of a small amount of NaOH. Conductivity measurements showed that the total concentration of salt was less than 0.001 N; *i.e.*, too small to cause the noted effect on the potential.) In the presence of salt, on the other hand, there is no effect on the potential but agglutination occurs in very high dilution with the immune serum and to a much less extent with the normal serum. This experiment shows that the effect of the serum on a suspension of bacteria in *concentrated salt* solution is not primarily on the charge but on the cohesive force. The serum raises the cohesive force and hence the critical charge to a value greater than the potential carried by the organism and they therefore agglutinate. The effect of the serum on the cohesion is shown in Fig. 6. The upper part of the figure shows that the addition of serum raises the cohesion to the value in distilled water; *i.e.*, it prevents the salt from decreasing the attractive force and thereby lowering the critical potential. The lower part of the figure shows the converse experiment; *i.e.*, the effect of salt on a film of washed, and of sensitized organisms. The salt decreases the cohesion of the washed organisms very markedly but has no effect on the cohesion of the film sensitized with serum.

The effect of varying both the salt and the serum concentration on the agglutination is shown in Table V. As the serum concentration is increased, the salt concentration in which complete agglutination occurs widens on both sides from 0.10 N. The lower limiting concentration of salt remains at about 0.01 N, however, and does not continue decreasing as the serum increases. In other words, the effect is not additive, but there is a critical concentration of serum beyond which there is little or no effect on the concentration of salt needed to agglutinate. This "critical" salt concentration corresponds to the point at which the charge on the organisms is about 10 millivolts; *i.e.*, just under the critical potential. This is the result expected if the

agglutinin forms a film on the surface of the organism. As soon as the layer is complete the addition of excess serum will have no effect. If this assumption is correct, it follows from Table V that agglutination occurs when the surface is about one-eighth covered. The inhibiting effect of strong salt solution is, as usual, due to the decrease in the cohesive force, when the organisms are not completely covered with immune body.

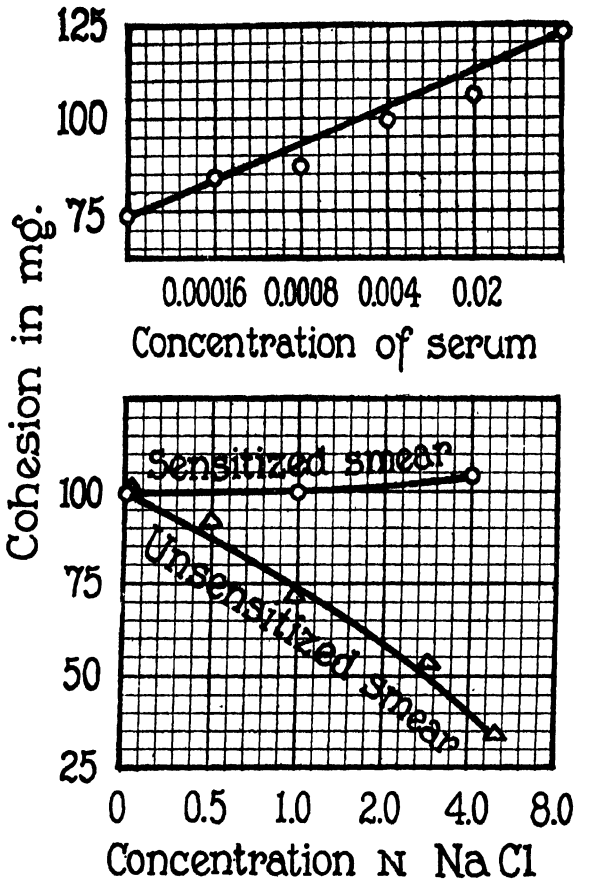

FIG. 6. Upper half, effect of the concentration of immune serum on the cohesive force between two films of *B. typhosus* in 0.10 N NaCl. Lower half, effect of concentration of NaCl on the cohesive force between, a, smears treated with immune serum and, b, untreated smears.

It is evident from the foregoing that the agglutination may be considered as caused by the salt, as Bordet stated. The serum, however, does not *sensitize* the bacteria but *protects* it from the salt so that the latter does not reduce the cohesive force. If we study the effect of salts and acids on the agglutination and charge of organisms sensitized with immune serum, we should expect then to obtain curves similar

TABLE V.

Influence of NaCl Concentration on Agglutination with Dialyzed Normal and Immune Serum.

Concentration of immune serum	Agglutination after 24 hrs. at 20°C.								
	Concentration of NaCl.								
	0	0.001	0.003	0.01	0.03	0.1	0.3	1.0	1.4
1:150	+	+	+++	C.	C.	C.	C.	C.	C.
1:300	−	−	+++	C.	C.	C.	C.	C.	C.
1:600	−	−	+++	C.	C.	C.	C.	C.	C.
1:1,200	−	−	++	C.	C.	C.	C.	C.	C.
1:2,400	−	−	+	C.	C.	C.	C.	C.	++
1:4,800	−	−	−	++	C.	C.	C.	C.	+
1:9,600	−	−	−	+	++	C.	C.	++	−
1:19,200	−	−	−	−	+	C.	++	+	−
0	−	−	−	−	−	+	+	−	−
Concentration of normal serum.									
1:12	+	++	+++	C.	C.	C.	C.	C.	C.
1:24	−	−	+	C.	C.	C.	C.	+++	+++
1:48	−	−	−	+++	+++	+++	++	+	+
1:96	−	−	−	++	+++	++	+	+	−
1:192	−	−	−	−	+	++	+	−	−
μ per sec.	−2.5	−1.3	−1.0	−0.7	−0.4	−0.15	0	0	0
Millivolt potential.	−32	−17	−13	−9.0	−5.6	−0.19	0	0	0

to those given in the preceding paper with the exception that the stable zone in high concentrations of salt would not appear and the agglutination should be found to depend entirely on the potential. A summary of a number of such experiments is given in Fig. 7. The serum concentration was 1:500 in all cases; *i.e.*, in excess. The figure shows that with the exception of strong acid solution, complete agglutination occurred whenever the potential was reduced below 15

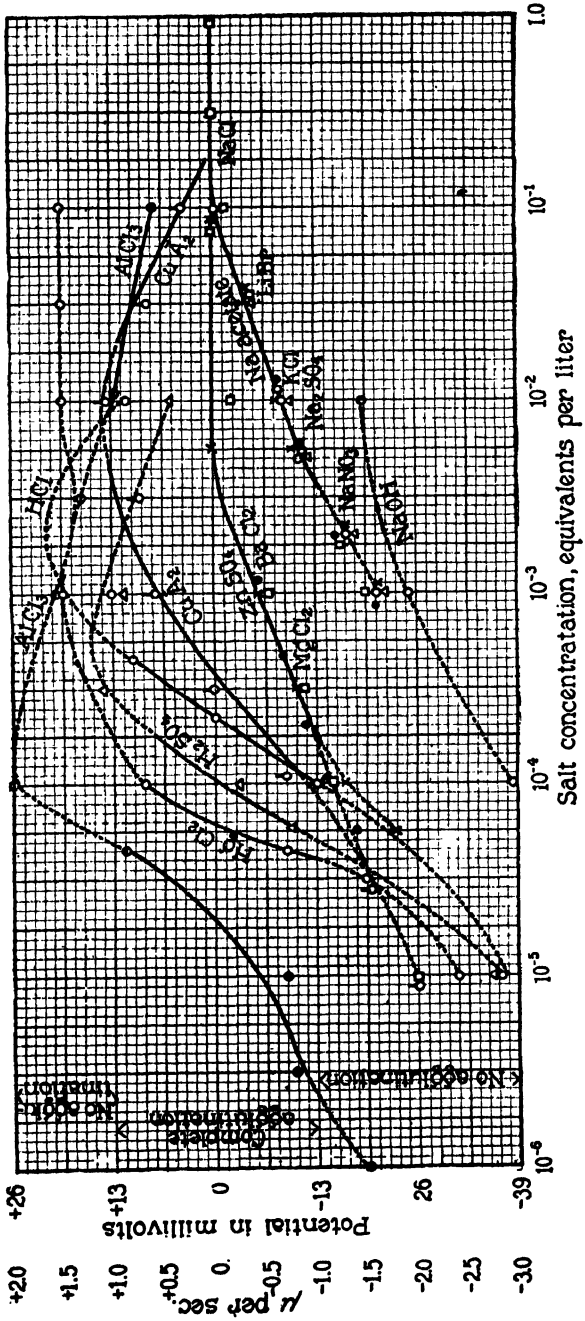

FIG. 7. Effect of the salt concentration on the agglutination and potential of *B. typhosus*, sensitized with immune serum.

millivolts, and there is no stable zone in concentrated salt. The effect of the strong acid is due partly to destruction of the antibody and partly to the fact that the combination of the antibody with the bacteria is less complete in acid solutions.[13]

The results also show that the effect of all monovalent cations (except hydrogen) was identical both as regards potential and agglutination. The valency and nature of the anion have no effect. This is the usual result when the particles are negative. The bivalent cations agglutinate in much lower concentration. The trivalent curves are not comparable owing to changes in the pH.

SUMMARY.

1. The addition of proteins or serum to suspensions of bacteria, (*Bacillus typhosus* or rabbit septicemia) at different pH widens the acid agglutination zone and shifts the isoelectric point to that of the added substance.

2. The amount of serum required to agglutinate is much less near the acid agglutination point of the organisms.

3. The addition of immune serum prevents the salt from decreasing the cohesive force between the organisms, and agglutination therefore is determined solely by the potential, provided excess immune body is present. Whenever the potential is decreased below 15 millivolts the suspension agglutinates.

[13] This point is taken up in the following paper.

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THE COMBINATION OF GELATIN WITH HYDROCHLORIC ACID.

BY DAVID I. HITCHCOCK.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 12, 1922.)

Titration curves for gelatin with different acids have been obtained by Loeb,[1,2] and used to show that the protein reacts stoichiometrically with the different acids. The amount of a strong acid combined with the protein may be obtained from the titration curve (in which the abscissæ are pH values and the ordinates concentrations of acid) by subtracting from the ordinates the amounts of acid necessary to bring the same volume of water, without protein, to the same pH values. In this way Loeb[2] obtained a curve for the amount of HCl combined with 1 gm. of gelatin at different pH values. Similar curves were obtained by Tague[3] for the combination of amino-acids with NaOH, and by the writer[4] for the combination of edestin with acids. Each of these combination curves appeared to become horizontal beyond a certain pH; *i.e.*, after enough acid (or alkali, in the case of Tague's experiments) had been added, the amount combined with the ampholyte became constant.

In a recent paper by Lloyd and Mayes[5] a curve is given to represent the amount of HCl combined with 1 gm. of gelatin. This curve, however, does not become horizontal, but rises with increasing acidity in a rather discontinuous manner. These authors did not obtain the amount of combined HCl in the way just described, but calculated it by the formula $N' = N - \frac{[H^+]\text{ corr.}}{\alpha}$ where $[H^+]\text{corr.} = \sqrt{[H^+] \times [Cl^-]}$,

[1] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 100.

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 50–51.

[3] Tague, E. L., *J. Am. Chem. Soc.*, 1920, xlii, 173.

[4] Hitchcock, D. I., *J. Gen. Physiol.*, 1921–22, iv, 597.

[5] Lloyd, D. J., and Mayes, C., *Proc. Roy. Soc., London, Series B*, 1922, xciii, 69.

N = normality of total HCl, α its degree of ionization, and N' = normality of combined HCl. The values which they used for the degree of ionization were conductivity ratios given by Lewis.[6] Inasmuch as the results of Lloyd and Mayes differed from those obtained by Loeb, it seemed worth while to repeat and amplify his experiments.

Solutions were made up containing 1, 2.5, and 5 gm. of gelatin in 100 cc. of HCl of various concentrations. The gelatin was taken from a stock solution of isoelectric gelatin which had been rendered practically ash-free in the way described by Loeb,[7] and was diluted to twice the concentration required for each set of experiments. 25 cc. samples of these solutions were measured out at 33°C. by a pipette, and each sample was diluted to 50 cc. by the addition of the proper amounts of 0.1 M or 1.0 M HCl and water from burettes. The concentration of the gelatin was checked by dry weight determinations of the amount of gelatin delivered by the 25 cc. pipette, and was accurate to about 1 part in 200. The concentration of the acid used was determined by titration against pure Na_2CO_3, and was accurate to 1 part in 500 or better. The pH of the solutions was determined at 33° with the hydrogen electrode and potentiometer, using rocking cells of the Clark type connected by a salt bridge of saturated KCl to a saturated KCl calomel cell. The pH values are referred to 0.1000 M HCl as a standard, its pH being taken as 1.036 at 33°. The E.M.F. readings obtained were reproducible to within 1 millivolt, which corresponds to about 0.02 pH.

The titration curves obtained in this way are given in Fig. 1, the abscissæ being pH values, and the ordinates total concentration of HCl expressed in millimoles per liter, which is the same as cc. of 0.1 M acid per 100 cc. The curve for 1 per cent gelatin represents three experiments, one of which was performed by Mr. M. Kunitz.

In order to determine how much of the total HCl was not combined with the gelatin, a series of solutions containing only HCl and water was prepared, and the pH values were determined. The values are given in Table I.

[6] Lewis, W. C. McC., A system of physical chemistry, London and New York, 3rd edition, 1920, ii, 219.

[7] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39; *J. Am. Chem. Soc.*, 1922, xliv, 213; Proteins and the theory of colloidal behavior, New York and London, 1922, 35.

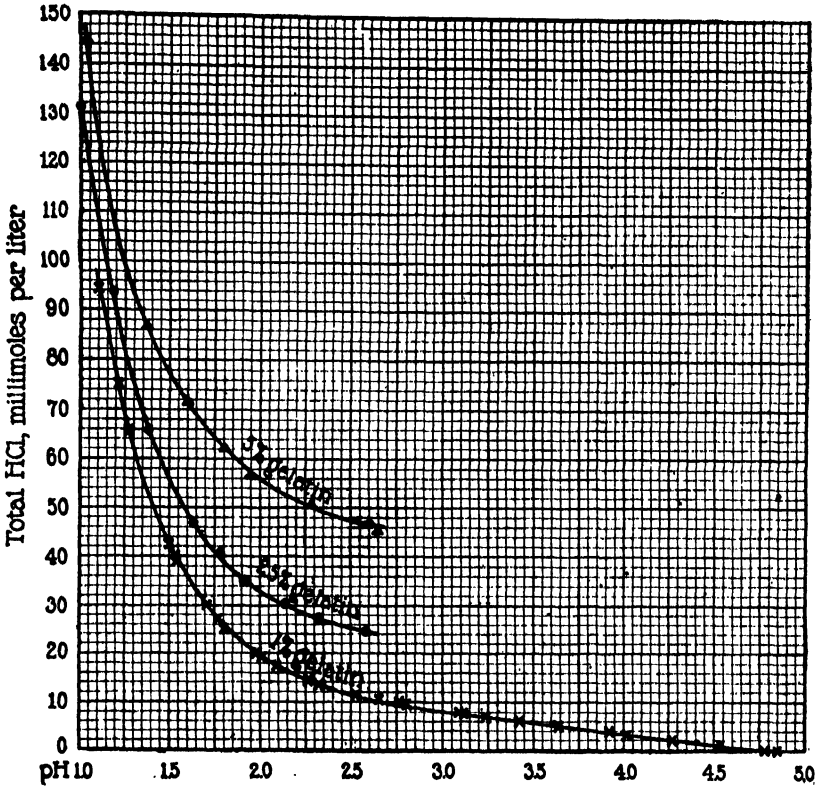
Total HCl, millimoles per liter
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
pH 1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0

FIG. 1.

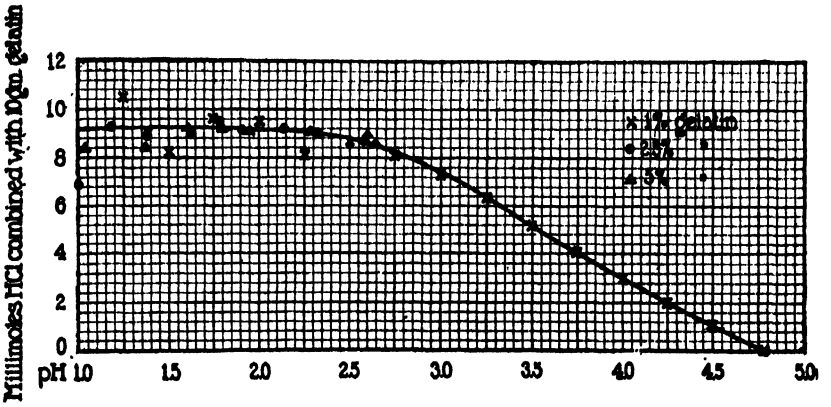
Millimoles HCl combined with 10gm. gelatin
12
10
8
6
4
2
0
pH 1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0

FIG. 2.

TABLE I.

Titration of Water with Hydrochloric Acid at 33°C.

Concentration of HCl, per liter, *millimoles*	0.05	0.10	0.19	0.39	0.68	0.97	1.95	3.89	8.75	15.56	24.31	48.63	100.00	117.7
pH	4.35	4.07	3.74	3.38	3.17	3.00	2.70	2.41	2.06	1.81	1.63	1.33	1.04	0.99

TABLE II.

Titration of Gelatin with Hydrochloric Acid.

pH of 1 per cent gelatin chloride*	1.25	1.50	1.75	2.00	2.25	2.50	2.75	3.00	3.25	3.50	3.75	4.00	4.25	4.50
Total HCl per liter, *millimoles*	69.5	41.3	27.6	19.5	14.7	11.8	9.8	8.3	6.8	5.5	4.3	3.1	2.0	1.1
Uncombined HCl	59.0	33.1	18.0	10.0	6.6	3.1	1.7	1.0	0.5	0.3	0.2	0.1	0.0	0.0
Combined HCl per 10 gm. gelatin, *millimoles*	10.5	8.2	9.6	9.5	8.1	8.7	8.1	7.3	6.3	5.2	4.1	3.0	2.0	1.1
pH of 2.5 per cent gelatin chloride	1.00	1.18	1.38	1.63	1.78	1.91	2.14	2.32	2.58					
Total HCl per liter, *millimoles*	131.3	93.8	65.7	46.9	40.8	35.0	30.2	27.2	24.3					
Uncombined HCl	114.0	70.5	43.5	24.3	16.7	12.3	7.3	4.8	2.6					
Combined HCl per 25 gm. gelatin, *millimoles*	17.3	23.3	22.2	22.6	24.1	22.7	22.9	22.4	21.7					
Combined HCl per 10 gm. gelatin, *millimoles*	6.9	9.3	8.9	9.0	9.5	9.1	9.2	9.0	8.7					
pH of 5 per cent gelatin chloride	1.03	1.37	1.61	1.80	1.95	2.28	2.60	2.64						
Total HCl per liter, *millimoles*	145.0	86.6	71.5	62.1	56.5	50.8	47.1	45.2						
Uncombined HCl	103.0	44.4	25.5	15.9	11.2	5.3	2.5	2.3						
Combined HCl per 50 gm. gelatin, *millimoles*	42.0	42.2	46.0	46.2	45.3	45.5	44.6	42.9						
Combined HCl per 10 gm. gelatin, *millimoles*	8.4	8.4	9.2	9.2	9.1	9.1	8.9	8.6						

* The figures for 1 per cent gelatin chloride were obtained from the curve averaging three experiments.

These results were plotted on a large scale and a smooth curve was drawn through the points. From this curve were read off the concentrations of free HCl present at the pH actually measured in each gelatin chloride solution, and these values were subtracted from the total HCl concentrations of the respective gelatin chloride solutions. The differences accordingly represent the millimoles of HCl combined with the amount of gelatin present in 1 liter, or the cc. of 0.1 M HCl combined with the gelatin present in 100 cc. These values were divided by the number of grams of gelatin in 100 cc. to get the cc. of 0.1 M HCl combined with 1 gm. of gelatin, which is the same as the number of millimoles of HCl combined with 10 gm. of gelatin. Table II indicates the method of calculation, and the final results are plotted in Fig. 2.

It will be noticed that the points lie fairly close to a smooth curve, except in the most acid region, where a small error in the pH may lead to a large error in the difference between the ordinates of two steep curves. The curve is horizontal between pH 1 and 2, indicating that here the gelatin is all combined with the acid. There is no evidence of the discontinuous sections found in the curve of Lloyd and Mayes.[5] This difference is due in part to differences in the experimental curves, but is also due largely to the method of calculation of the combined acid. The method used by Lloyd and Mayes involves the assumption that the uncombined HCl is ionized to the extent indicated by the conductivity ratio for a different concentration of HCl; namely, the concentration of the total HCl present. Moreover, these authors have neglected the difference between the conductivity ratios and the activity coefficients or hydrogen electrode values for HCl, which is clearly brought out by the table given by Lewis[6] from which they obtained their ionization values. The method of calculation used by Loeb, Tague, and the present writer involves the assumption that the same concentration of uncombined HCl is necessary to furnish the same hydrogen ion concentration, as determined by the hydrogen electrode, whether or not gelatin is present. The latter assumption seems to lead to more reasonable results.

The maximum height of the curve in Fig. 2, 9.2 millimoles of HCl for 10 gm. of gelatin, indicates that a 1 per cent gelatin solution has a

normality of 0.0092 with respect to its combination with HCl, or that the combining weight of gelatin is $\frac{10}{0.0092}$, or about 1,090. While the exact height of the maximum is still more or less uncertain, it is probable that this value of the combining weight is more nearly correct than the smaller values given by Procter,[8] Wilson,[9] Wintgen and Krüger,[10] and Wintgen and Vogel,[11] because the calculation involves simpler and more probable assumptions. Moreover, the earlier workers did not have ash-free or isoelectric gelatin at their disposal.

It is possible to calculate from these results an ionization constant for gelatin, assuming it to react as a mono-acid base. The simplest way of doing this is based on the resemblance between the combination curve and the dissociation curve of a base. The equation for the ionization of such a base is $\alpha = \frac{k_b}{k_b + [OH^-]}$ where α represents the fraction ionized and k_b the ionization constant. Since the ion product of water, k_w, is equal to $[H^+] \times [OH^-]$, this becomes

$$\alpha = \frac{[H^+]}{[H^+] + K}, \text{ if } K = \frac{k_w}{k_b}$$

Michaelis[12] has pointed out that if α is plotted against log $[H^+]$, at the point where $\alpha = \frac{1}{2}$, log $[H^+]$ = log K. Applying this to Fig. 2, $\alpha = \frac{1}{2}$ where the ordinate = 4.6 or pH = pK = 3.625. Accordingly $K = 2.4 \times 10^{-4}$. This is of the same order as the value obtained by Procter and Wilson[13] and is intermediate between the values obtained by Wintgen and Krüger[10] and Wintgen and Vogel,[11] but differs by a whole power of 10 from that obtained by Lloyd and Mayes.[5] Inasmuch as the use of this constant leads to only rough agreement with the combination curve in Fig. 2, it is not desired to lay any stress on this calculation. It is quite certain that gelatin is not a mono-acid base, though its combination curve may resemble the ionization curve of such a base.

[8] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313.
[9] Wilson J. A., *J. Am. Leather Chem. Assn.*, 1917, xii, 108.
[10] Wintgen, R., and Krüger, K., *Kolloid-Z.*, 1921, xxviii, 81.
[11] Wintgen, R., and Vogel, H., *Kolloid-Z.*, 1922, xxx, 45.
[12] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914, 20.
[13] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

SUMMARY.

The amount of HCl combined with a given weight of gelatin has been determined by hydrogen electrode measurements in 1 per cent, 2.5 per cent, and 5 per cent solutions of gelatin in HCl of various concentrations, by correcting for the amount of HCl necessary to give the same pH to an equal volume of water without protein. The curve so obtained indicates that the amount of HCl combined with 1 gm. of gelatin is constant between pH 1 and 2, being about 0.00092 moles.

The writer wishes to express his gratitude for the advice of Dr. Jacques Loeb, under whose direction this work was done.

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THE MECHANISM BY WHICH TRIVALENT AND TETRAVALENT IONS PRODUCE AN ELECTRICAL CHARGE ON ISOELECTRIC PROTEIN.

BY JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 4, 1922.)

I.

Experiments on anomalous osmosis recently published[1] suggest that isoelectric gelatin assumes a negative charge when a solution of a salt with tetravalent anion, *e.g.* $Na_4Fe(CN)_6$, is added; and that isoelectric gelatin assumes a positive charge when a salt with a trivalent cation, *e.g.* $LaCl_3$, is added. In these experiments special care was taken that the pH of the solutions was that of the isoelectric point of gelatin; *i.e.*, pH 4.7. $CaCl_2$, Na_2SO_4, and NaCl had apparently no such effect on the charge of isoelectric gelatin.

The question arose whether it was possible to support this suggestion by direct measurements of the P.D. between isoelectric solid gelatin and the surrounding salt solution of pH 4.7; and if this was the case what was the reason for this peculiar action of trivalent and tetravalent ions.

The method of the experiments was as follows: Doses of 1 gm. of powdered gelatin (going through mesh 30 but not through 60) were rendered isoelectric in the way described in previous publications. They were then put for 2 hours at 15°C. into 200 cc. of solutions of $LaCl_3$ of different concentration, all of pH 4.7. In this time equilibrium was practically established between the gel and the outside $LaCl_3$ solution. The suspension was then transferred into cylinders and the gelatin granules allowed to settle (at 15°C.). The supernatant liquid and the solid gelatin granules were then separated by filtration, the solid gelatin granules were melted by warming, poured into cylinders with two glass tubes attached (as described in

[1] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 463.

a recent book[2]) and the gelatin was allowed to solidify at 15°C. The P.D. between the solid gelatin and the supernatant $LaCl_3$ solution was then measured with two identical saturated KCl-calomel electrodes connected with a Compton electrometer as previously described.[3] Table I gives the observed P.D. The $LaCl_3$ solutions used varied between M/24 and M/98,304.

The gelatin was nearly but not entirely isoelectric, since it had a positive charge of about 15 millivolts. This means that its pH was not 4.7 but probably about 4.6 or possibly slightly less.

Table I shows that the P.D. rises upon the addition of increasing quantities of $LaCl_3$ from 15 to 23.5 millivolts at a concentration of M/6,144 $LaCl_3$. When the concentration of $LaCl_3$ rises beyond this point, the P.D. decreases again until it becomes 5.0 at a concentration of M/24 $LaCl_3$. $LaCl_3$ acts, therefore, upon the P.D. of isoelectic gelatin in a similar way to HCl. In the case of HCl it has been proven that a salt is formed between gelatin (or any other isoelectric protein) in which the H ion becomes part of a complex positively charged protein ion while the Cl is the anion. It is natural to assume that the reaction is similar to that between NH_3 and HCl where the salt NH_4Cl is formed, inasmuch as the protein contains NH_2 or NH groups in which the N is still able to attract and hold electrostatically another H ion. The correctness of this view is supported by the fact that when we add HCl to a 1 per cent solution of gelatin and measure the Cl potential of the solution, the Cl potential is the same as when we add the same amount of HCl to the same quantity of water (without gelatin), while the H potential is considerably lower in the gelatin solution than in the pure aqueous solution.

When we add increasing concentrations of HCl to solid isoelectric gelatin, gelatin chloride is formed and the ionization of the protein leads to the establishment of a Donnan equilibrium between the solid gelatin and the surrounding aqueous solution, and the unequal distribution of the oppositely charged ions inside and outside the gel is responsible for the P.D.[3] The theory of membrane equilibria

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 154.

[3] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 120 ff; *J. Gen. Physiol.*, 1920–21, iii, 667; 1921–22, iv, 351, 463.

demands that the P.D. increase with the increase in the concentration of gelatin ions formed, *i.e.* with the increase in the concentration of the acid added, and that the P.D. diminish with the increase of the concentration of Cl ions. At first, the augmenting influence of the acid (through formation of gelatin chloride) increases more rapidly with increasing concentration of acid than the depressing effect of the Cl ions, until a certain part of the gelatin is transformed into ions. From that point on the increase in P.D. due to the ionization of the gelatin increases less rapidly than the depressing effect of the Cl ions and the P.D. diminishes again when more acid is added. This has all been discussed more fully and need not be repeated here.[3]

The experiments mentioned in Table I show that $LaCl_3$ acts in a way entirely similar to HCl. When $LaCl_3$ solutions of low concentration (and of pH 4.7) are added to isoelectric gelatin, the P.D. rises with increasing concentration of $LaCl_3$ until the concentration is M/6,144, when the P.D. falls again. This suggests that the La ion of the $LaCl_3$ solution enters into combination with gelatin, as does the H in the case of HCl, by giving rise to gelatin-lanthanum chloride, in which the cation is a complex positively charged gelatin-La ion, while the anion is the Cl ion. The increase in ionization of the gelatin causes an increase in the P.D., while the Cl ion depresses the P.D. just as if HCl had been added. There is this difference between the gelatin chloride formed by the reaction between isoelectric gelatin and HCl and the gelatin chloride formed by the reaction between gelatin and $LaCl_3$; namely, that the $LaCl_3$ can be easily washed away while the HCl is held more tenaciously.[4] This is easily understood, since the radius of the La ion is so large that in spite of the high charge of the ion it is held with a considerably smaller force by the gelatin than is the H ion, which has no shell of electrons and whose positive nucleus can approach the N of the gelatin very closely.

The action of solutions of $Na_4Fe(CN)_6$ of pH 4.7 on isoelectric gelatin is similar to that of NaOH. The method was the same as that described in the case of $LaCl_3$. The results are given in Table II, showing that $Na_4Fe(CN)_6$ charges the gelatin negatively, the charge

[4] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 27; *J. Gen. Physiol.*, 1918–19, i, 237.

TABLE I.

P.D. *between Solid Isoelectric Gelatin and Solutions of $LaCl_3$ at pH near (but Probably Slightly Below) 4.7. Solid Gelatin Positively Charged.*

Concentration of $LaCl_3$.	0	M/98,304	M/49,152	M/24,576	M/12,288	M/6,144	M/3,072	M/1,536	M/768	M/384	M/192	M/96	M/48	M/24
P. D., *millivolts* ...	15.0	16.0	20.0	20.0	21.0	23.5	22.5	22.0	17.5	14.0	10.0	9.0	6.0	5.0

TABLE II.

P.D. *between Solid Isoelectric Gelatin and $Na_4Fe(CN)_6$ at pH near 4.7. Solid Gelatin Is Negatively Charged Where Minus Sign Is Added.*

Concentration of $Na_4Fe(CN)_6$.	0	M/32,768	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
P. D., *millivolts*.....	15.0	−3.0	−9.0	−15.0	−22.0	−13.0	−13.0	−11.0	−8.5	−5.5	−4.0	−2.5	−1.5	−1.0

TABLE III.

Influence of Different Concentrations of NaCl, Na_2SO_4, and $CaCl_2$ on the P.D. *between Solid Gelatin and Outside Solution. Gelatin Always Positively Charged unless Minus Sign Is Added.*

Concentration.	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1M
	millivolts	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*
NaCl.....	13.0		12.0	9.0	7.0	4.0	2.0	1.0	0	0	0	0	0	0.5	1.0
$CaCl_2$.....			13.0	6.0	9.0	2.5	3.5	3.0	2.0	1.5	1.5	3.0	2.5	2.5	1.0
Na_2SO_4.....		7.0	4.5	0.5	−0.5	−2.0	−2.0	−2.0	−1.5						

increasing with an increase in the concentration of $Na_4Fe(CN)_6$ until the concentration of M/4,096 is reached where the P.D. between solid gelatin and $Na_4Fe(CN)_6$ solution is 22 millivolts, the gelatin having the negative charge. Upon increasing the concentration of $Na_4Fe(CN)_6$ beyond M/4,096 the P.D. diminished again until it became 1 millivolt when the concentration of $Na_4Fe(CN)_6$ was M/8 (Table II).

The next step was to find whether solutions of $CaCl_2$ of pH 4.7 also give a positive charge to isoelectric gelatin and whether solutions of Na_2SO_4 give a negative charge. Table III shows that both of these salts, as well as NaCl, have only a depressing effect on the potential of isoelectric gelatin but cause no increase of P.D. in low concentration.

There is, however, a slight difference between the action of the three salts. While in the case of Na_2SO_4 gelatin becomes slightly negative in concentrations beyond M/512, this does not happen in the case of NaCl and $CaCl_2$. The negative charge is, however, so small that it would not be wise to attribute much importance to this fact. The important fact is that solutions of $LaCl_3$ and $Na_4Fe(CN)_6$ of pH 4.7 have a similar influence on isoelectric gelatin to that of acids and alkalies respectively, while this effect cannot be demonstrated in the case of NaCl, $CaCl_2$, or Na_2SO_4. This would be intelligible on the basis of the assumption that the electrostatic attraction of Na, Cl, SO_4, and Ca ions is not sufficient to cause the formation of any considerable quantity of ionizable gelatin salts when reacting with isoelectric gelatin; at least not in the concentrations of salts used in this experiment. If this surmise is correct, we must consider the fact that the electrostatic attraction of an ion is a function of at least two variables; namely, the number of charges and the radius. If this is taken into consideration it is not to be expected that all divalent ions should be as inactive as Ca or SO_4.

II.

If the influence of $LaCl_3$ and $Na_4Fe(CN)_6$ on the P.D. of solid isoelectric gelatin is due to ionization of the protein and the establishment of a Donnan equilibrium as a consequence of this ionization, this should betray itself also in the osmotic pressure of mixtures of

isoelectric gelatin in solutions of $LaCl_3$ or $Na_4Fe(CN)_6$ of pH 4.7. The osmotic pressure of 1 gm. of isoelectric gelatin in 100 cc. of solution should at first increase with the concentration of $LaCl_3$ or $Na_4Fe(CN)_6$ until a maximum is reached, and the osmotic pressure should then drop again if the concentration of these two salts is increased beyond this point. On the other hand, NaCl, LiCl, $MgCl_2$, $CaCl_2$, or Na_2SO_4 should have either no such effect, or it should be considerably smaller.

A stock solution of concentrated isoelectric gelatin was prepared and so diluted that 1 gm. (by dry weight) of such isoelectric gelatin was contained in 100 cc. of H_2O or various concentrations of the salts mentioned, all of a pH of 4.7. Collodion bags of a volume of about 50 cc. were filled with these gelatin solutions. Each bag was put into a beaker containing 350 cc. of a solution of the same salt and the same concentration as that in which the gelatin was dissolved. These 350 cc. of outside solutions were free from gelatin but had the same pH as the salt solution inside the bag; namely, 4.7. The osmotic pressure was measured in the way previously described and the final measurements were made after 18 or 20 hours. The temperature was kept constant, in some cases at 24°, but generally at 27°C. The higher temperature was chosen to prevent the gelatin from setting to a gel too quickly; *i.e.*, before osmotic equilibrium between the gelatin solution and the outside solution was established. Isoelectric gelatin is rather insoluble but becomes more soluble if salt is added. We shall discuss this more fully in a later part of this paper.

The results of these experiments are given in Table IV. From this table it is clear that the addition of $LaCl_3$ and $Ce(NO_3)_3$ or $Na_4Fe(CN)_6$ acts on the osmotic pressure of 1 per cent solutions of isoelectric gelatin in a similar way as the addition of acid or alkali, inasmuch as the addition of little $LaCl_3$ or $Ce(NO_3)_3$ or $Na_4Fe(CN)_6$ raises the osmotic pressure until a maximum is reached at a concentration of M/2,048 for $LaCl_3$ and of M/4,096 for $Na_4Fe(CN)_6$; while the addition of more $LaCl_3$ or $Ce(NO_3)_3$ or $Na_4Fe(CN)_6$ depresses the osmotic pressure. None of the other salts used, NaCl, LiCl, $MgCl_2$, $CaCl_2$, or Na_2SO_4, acted in this way. There was possibly a slight rise when the concentration of these latter salts became very high.

These experiments confirm the conclusion reached in connection with the experiments on the charge of solid gelatin that salts with trivalent cations or tetravalent anions ionize isoelectric gelatin and thereby set up a Donnan equilibrium; while salts of the type of $CaCl_2$ or Na_2SO_4 or NaCl do not act this way.

In the experiments just described it seemed advisable to measure also the influence of different salt solutions of pH 4.7 on the P.D. between solutions of isoelectric gelatin inside the collodion bag and the outside aqueous solution free from gelatin but also of pH 4.7. These measurements were taken at the end of the experiments after

TABLE IV.

Influence of Salt Solutions of pH 4.7 on the Osmotic Pressure (in Mm. H_2O) of 1 Per Cent Solutions of Isoelectric Gelatin.

Concentration.	0	M/65,536	M/32,768	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M	2 M
$Na_4Fe(CN)_6$	18			51	94	95	90	75	63	51	48	41	43	38					
Na_2SO_4	30						27	25	30	29	33	32	26	32	33	29			
NaCl	23	22	22	23	23	22	23	27	28	31	25	29	26	28	28	30	35	38	
LiCl							26	26	28	27	30	28	32	36	38	39	40	43	
$MgCl_2$		23	20	24	27	23	28	24	28	28	27	29	33	31	33	36	40	43	42
$CaCl_2$		22	25	24	26	28	26	30	32	29	27	32	30	31	33	40	38	38	40
$LaCl_3$	27	26	32	40	51	52	60	62	57	57	54	50	48						
$Ce(NO_3)_3$	23	28	34	46	50	58	71	69	63	55	50	45							

osmotic equilibrium was established. The figures show that the addition of little $LaCl_3$ or $Ce(NO_3)_3$ increases the P.D. across the membrane until a maximum is reached at a concentration near M/8,192 or M/4,096 and that the P.D. drops again with a further increase in the concentration of the salt. The gelatin is positively charged, showing that the gelatin forms cations. $Na_4Fe(CN)_6$ acts similarly except that gelatin is negatively charged. NaCl, LiCl, Na_2SO_4, $MgCl_2$, and $CaCl_2$ create no potential difference; or in other words, they cause no ionization of isoelectric gelatin. These results agree with the results obtained with solid gelatin and they agree also with the results obtained in the experiments on anomalous osmosis (Table V).

TABLE V.

Influence of Salt Solutions of pH 4.7 on Membrane Potentials in Millivolts between Solutions of Isoelectric Gelatin and Aqueous Solutions Free from Gelatin. Gelatin Solution Positive unless Minus Sign Is Added.

Concentration.	0	M/65,536	M/32,768	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M	2 M
$Na_4Fe(CN)_6$	−3.0			−9.0	−13.5	−10.0	−6.0	−4.0	−3.0	−1.5	0	0	−0.5	−0.5					
Na_2SO_4	−1.0						−0.5	0	−0.5	0	0	0	0	0	0	0	0		
NaCl	1.0			3.0	2.0	0.5	1.0	0.5	0	0	−0.5	0	0	−0.5	0	0	0	0	
LiCl	0.5						0	−1.0	−0.5	0	0	0	0	−0.5	0	0	0	0	
$MgCl_2$				0	0	0	0	0	0	0	0	0	0	0	−0.5	−0.5	−1.0	−1.5	0
$CaCl_2$	−1.0			0.5	1.0	1.5	1.5	0.5	0.5	0.5	0	0	0	0	0	−0.5	−0.5	−0.5	−1.0
$LaCl_3$	−0.5	1.0	3.0	5.0	8.0	5.0	4.0	3.5	3.0	2.0	1.5	1.0	1.0						
$Ce(NO_3)_3$	−2.5	1.0	5.0	6.5	6.0	7.5	7.0	5.0	3.5	2.0	1.0	0.5							

TABLE VI.

Influence of Solutions of $LaCl_3$ on pH of 1 Per Cent Solutions of Nearly Isoelectric Gelatin.

Concentration of $LaCl_3$	0	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64
pH	4.85	4.80	4.78	4.70	4.64	4.57	4.52	4.45	4.39	4.34

III.

While all this seems clear, there remains the possibility that the effects of $LaCl_3$ and of $Na_4Fe(CN)_6$ on the P.D. and osmotic pressure of isoelectric gelatin are due to a different kind of ionization from that assumed above. When a solution of $LaCl_3$ of pH 4.7 is added to a gelatin solution of the same pH, the solution becomes more acid; and when a solution of $Na_4Fe(CN)_6$ of pH 4.7 is added to a solution of isoelectric gelatin, the solution becomes more alkaline. A solution of $LaCl_3$ of pH 4.7 makes, therefore, isoelectric gelatin more acid and this should lead to a formation of gelatin chloride, without the necessity of assuming the formation of a complex gelatin-La cation; while solutions of $Na_4Fe(CN)_6$ of pH 4.7 make isoelectric gelatin more alkaline and this should lead to a formation of Na gelatinate. To test this possibility 1 per cent solutions of nearly isoelectric gelatin (pH 4.85) were made up in various concentrations of $LaCl_3$ of pH 4.7 and the pH was determined electrometrically (Table VI).

According to Table V the highest P.D. is reached in $LaCl_3$-gelatin mixtures at a concentration of $LaCl_3$ of M/8,192; such a solution influences the pH of the gelatin solution too little to account for the P.D. of 8.0 millivolts, since near the isoelectric point the difference between pH inside and outside is almost zero.

It was then attempted to repeat the experiments on the influence of $Ce(NO_3)_3$ on the P.D. and osmotic pressure of solutions of isoelectric gelatin in buffer solutions, but these experiments failed since the addition of the salt contained in the buffer solution in itself sufficed to suppress the P.D. to be expected.

To reach a decision the following experiments were made.

1 per cent solutions of isoelectric gelatin were prepared and to some solutions was added HCl to others NaOH so that the pH values obtained were approximately 3.5, 4.0, 4.4, 4.7, 5.0, and 5.5. These gelatin solutions were made up in solutions of different salts of the same pH. In this way it was possible to ascertain the influence of these salts on the pH of the gelatin solution (Table VII).

The P.D. between these gelatin solutions inside the collodion bags and the outside aqueous solutions of the same salt and originally

of the same pH as the gelatin solution (but without gelatin), was also measured after osmotic equilibrium was established. The figures for the P.D. are always under the figures for the pH of the gelatin-salt solutions. The result is very striking. The gelatin is always positively charged when the gelatin is dissolved in M/768 $LaCl_3$ regardless of the pH. Even when the pH of the solution is on the alkaline side of the isoelectric point the gelatin is positively charged in the presence of M/768 $LaCl_3$. No such result is observed when the salt added is NaCl, $CaCl_2$, or Na_2SO_4. In this case the P.D. across the membrane was zero at pH 4.7 and 4.8 and the gelatin became negative when the pH became 5.0 or above. This shows that the positive charge of isoelectric gelatin in the presence of

TABLE VII.

		Gelatin-acid salts.			Isoelectric gelatin.	Metal gelatinates.	
M/768 $LaCl_3$.	pH	3.66	3.98	4.32	4.72	4.82	5.3
	P.D., *millivolts*	+8.5	+7.0	+5.5	+3.5	+3.0	+2.0
M/512 $CaCl_2$.	pH	3.62	4.02	4.44	4.72	5.02	5.43
	P.D., *millivolts*	+8.5	+5.5	+2.5	0	−1.0	−2.5
M/256 NaCl.	pH	3.66	3.98	4.49	4.80	5.19	5.68
	P.D., *millivolts*	+9.5	+7.0	+2.5	0	−3.0	−6.0
M/512 Na_2SO_4.	pH	3.63	4.06	4.41	4.83	5.16	5.82
	P.D., *millivolts*	+6.0	+4.0	+1.0	0	−2.0	−3.0

$LaCl_3$ of pH 4.7 cannot be due to the change in pH but must be due to some other cause of ionization of gelatin such as the formation of a complex gelatin-La cation.

The pH for $Na_4Fe(CN)_6$ could not be measured electrometrically.

In view of all these results we must draw the conclusion that salts with trivalent (and probably also tetravalent) cations cause isoelectric gelatin and gelatin on the alkaline side of the isoelectric point (but near this point) to assume a positive charge owing to the formation of positively charged protein ions, probably of the type gelatin-La. This explains the reversal of the charge of gelatin by trivalent cations observed in anomalous osmosis and kindred phenomena. Tetravalent anions confer a negative charge on isoelectric gelatin and the mechanism is probably similar. Changes of

hydrogen ion concentration may enter to some extent into these effects but they are probably of only secondary importance. No such effects have thus far been obtained with salts possessing divalent or monovalent ions; *i.e.*, salts of the type Na_2SO_4, $CaCl_2$, and NaCl.

IV.

In a gelatin chloride solution of pH 3.0, the greater part of the gelatin is ionized, and since the H ion is held more firmly by the gelatin than the La ion, the addition of $LaCl_3$ to a solution of gelatin chloride of pH 3.0 should not have any augmenting effect on the P.D. or the osmotic pressure of the gelatin chloride solution. The only effect the addition of $LaCl_3$ to a solution of gelatin chloride should have is the depressing effect of the Cl ions. Hence when we mix a solution of gelatin chloride of pH 3.0 with solutions of $LaCl_3$ of the same pH the effect on the P.D. and the osmotic pressure should be merely a depression of the values of these latter properties, and the depression should be quantitatively identical with the effect of $CaCl_2$ or NaCl solutions of the same concentration of Cl ions and the same pH.

This fact had already been ascertained in previous experiments already published,[3] but since these direct measurements of P.D. are of such importance for the theory of the origin of the P.D. between colloids and aqueous solutions it seemed advisable to repeat them. 1 gm. of gelatin chloride of pH 3.0 was dissolved in 100 cc. of solutions of various salts all brought to pH 3.0 through the addition of HCl. Collodion bags were filled with these solutions of gelatin chloride in different salts and each bag was dipped into 350 cc. of an aqueous solution of the same concentration of the same salt and the same pH as that inside the collodion bag, but without gelatin. Water diffused into the collodion bag until osmotic equilibrium was established, and the next day the final osmotic pressure and the P.D. between gelatin solution and outside aqueous solution were measured. The solutions of the three salts were prepared in such a way as to have the same concentration of Cl. In Fig. 1 are plotted the P.D. as ordinates over the concentration of the Cl ions as abscissæ. It is obvious that the influence of NaCl, $CaCl_2$, and $LaCl_3$

on the P.D. is identical for the same concentration of Cl. This proves that only the Cl ion of these salts influences the P.D. of a gelatin chloride solution of pH 3.0 and that the La ion does not increase the P.D. of the solution.

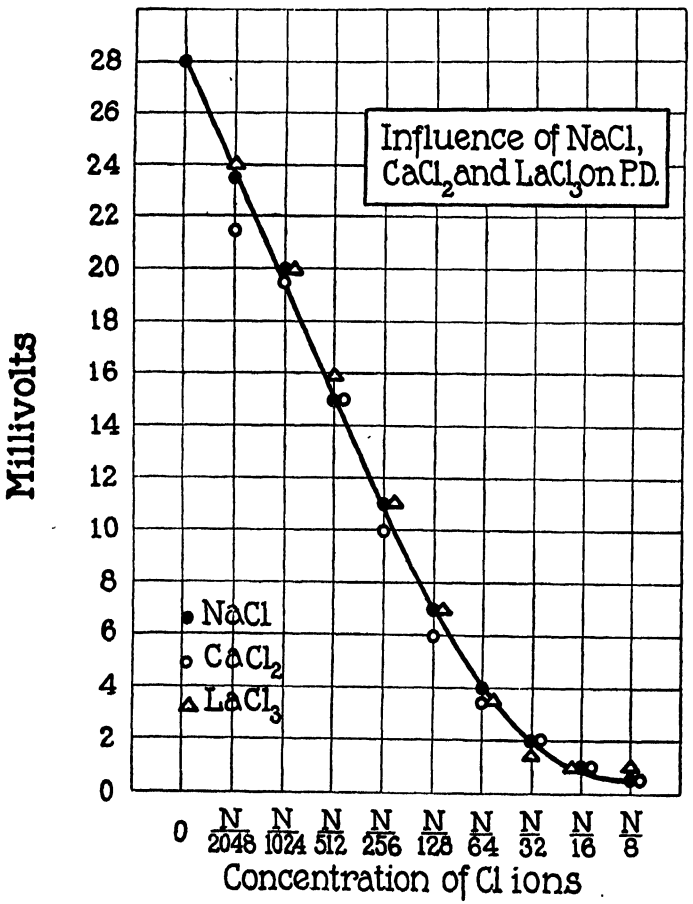

FIG. 1. Depressing influence of NaCl, $CaCl_2$, and $LaCl_3$ on the P.D. between a 1 per cent solution of gelatin chloride of pH about 3.0 and aqueous solutions of the salts originally of the same pH, the two solutions being separated by collodion membranes. Ordinates are the P.D., abscissæ the concentrations of Cl ions of the salts. The depressing effect of the three salts is the same for the same concentration of Cl ions, proving that the cations do not influence the P.D. of gelatin chloride solutions.

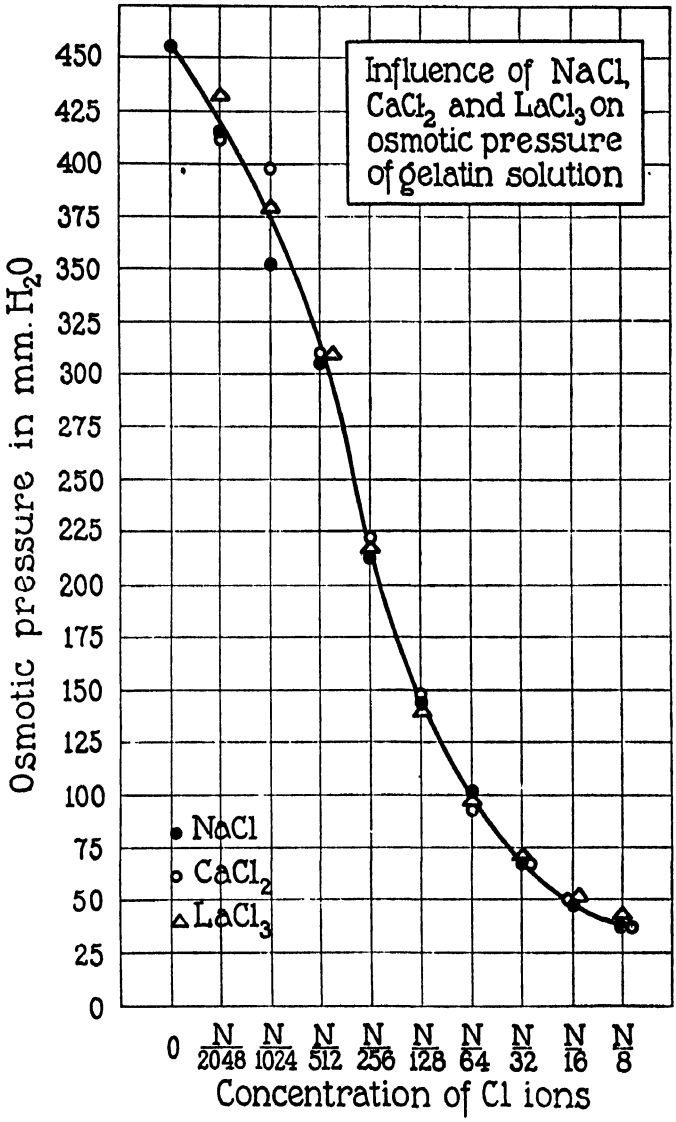

FIG. 2. Influence of NaCl, $CaCl_2$, and $LaCl_3$ on osmotic pressure of a 1 per cent solution of gelatin chloride of pH 3.0. Ordinates are osmotic pressure in mm. H_2O, abscissæ the concentrations of Cl ions of the salts. The depressing effect is the same for the three salts, proving that only the anion influences the osmotic pressure in this case.

TABLE VIII.

Influence of NaCl on the P.D. *of 1 Per Cent Solutions of Gelatin Chloride, pH 3.0.*

Concentration of NaCl.	0	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
pH inside	3.35	3.29	3.25	3.21	3.15	3.09	3.06	3.05	3.05	3.06
pH outside	2.88	2.89	2.90	2.93	2.95	2.96	2.98	3.01	3.02	3.05
pH inside minus pH outside	0.47	0.40	0.35	0.28	0.20	0.13	0.08	0.04	0.03	0.01
P.D. calculated, *millivolts*	+28.0	+23.5	+20.5	+16.5	+12.0	+7.5	+4.5	+2.5	+1.8	+0.6
P.D. observed, *millivolts*	+28.0	+23.5	+20.0	+15.0	+11.0	+7.0	+4.0	+2.0	+1.0	+0.5

TABLE IX.

Influence of $CaCl_2$ on the P.D. *of 1 Per Cent Solutions of Gelatin Chloride, pH 3.0.*

Concentration of $CaCl_2$.	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16
pH inside	3.34	3.30	3.24	3.20	3.13	3.09	3.06	3.04	3.04	3.03
pH outside	2.88	2.90	2.91	2.94	2.93	2.96	2.99	2.99	3.01	3.01
pH inside minus pH outside	0.46	0.40	0.33	0.26	0.20	0.13	0.07	0.05	0.03	0.02
P.D. calculated, *millivolts*	+27.0	+23.5	+19.5	+15.5	+11.5	+7.5	+4.0	+3.0	+1.8	+1.2
P.D. observed, *millivolts*	+28.0	+21.5	+19.5	+15.0	+10.0	+6.0	+3.5	+2.0	+1.0	+0.5

TABLE X.

Influence of $LaCl_3$ on the P.D. *of 1 Per Cent Solutions of Gelatin Chloride, pH 3.0.*

Concentration of LaCl₃	0	M/6,144	M/3,072	M/1,536	M/768	M/384	M/192	M/96	M/48	M/24
pH inside	3.33	3.27	3.23	3.18	3.12	3.08	3.06	3.03	3.03	3.02
pH outside	2.88	2.87	2.90	2.93	2.95	2.97	3.01	3.00	3.01	3.01
pH inside minus pH outside	0.45	0.40	0.33	0.25	0.17	0.11	0.05	0.03	0.02	0.01
P.D. calculated, *millivolts*	+27.0	+24.0	+20.0	+15.0	+10.0	+6.5	+3.0	+2.0	+1.0	+0.5
P.D. observed, *millivolts*	+28.0	+24.0	+20.0	+16.0	+11.0	+7.0	+3.5	+1.5	+1.0	+1.0

In these experiments the hydrogen ion concentrations of the inside and outside solutions were measured and the writer begs leave to give these results (Tables VIII, IX, and X), since they prove once more that the P.D. between a solution of gelatin chloride and an outside solution across a collodion membrane is determined by the difference in the pH in the inside and outside solutions, as the Donnan theory demands. In these experiments the influence of the three salts on the osmotic pressure of the gelatin chloride solutions was also measured and the results are given in Fig. 2 showing that the osmotic pressure of a gelatin chloride solution of pH 3.0 is influenced only by the anion but not by the cation of the salt, since the effect of the three salts on the osmotic pressure of the solution is exactly the same when plotted over the concentration of the Cl ions. At this pH, therefore, the La does not increase the osmotic pressure of gelatin chloride solutions.

These observations support the idea that trivalent and tetravalent ions are able to transfer their charge to isoelectric protein by causing the protein to be ionized; probably in such a way that the trivalent or tetravalent ion is part of a complex protein ion.

SUMMARY AND CONCLUSIONS.

1. Experiments on anomalous osmosis suggested that salts with trivalent cations, *e.g.* $LaCl_3$, caused isoelectric gelatin to be positively charged, and salts with tetravalent anions, *e.g.* $Na_4Fe(CN)_6$, caused isoelectric gelatin to be negatively charged. In this paper direct measurements of the P.D. between gels of isoelectric gelatin and an aqueous solution as well as between solutions of isoelectric gelatin in a collodion bag and an aqueous solution are published which show that this suggestion was correct.

2. Experiments on anomalous osmosis suggested that salts like $MgCl_2$, $CaCl_2$, NaCl, LiCl, or Na_2SO_4 produce no charge on isoelectric gelatin and it is shown in this paper that direct measurements of the P.D. support this suggestion.

3. The question arose as to the nature of the mechanism by which trivalent and tetravalent ions cause the charge of isoelectric proteins. It is shown that salts with such ions act on isoelectric gelatin in a way

similar to that in which acids or alkalies act, inasmuch as in low concentrations the positive charge of isoelectric gelatin increases with the concentration of the $LaCl_3$ solution until a maximum is reached at a concentration of $LaCl_3$ of about M/8,000; from then on a further increase in the concentration of $LaCl_3$ diminishes the charge again. It is shown that the same is true for the action of $Na_4Fe(CN)_6$. From this it is inferred that the charge of the isoelectric gelatin under the influence of $LaCl_3$ and $Na_4Fe(CN)_6$ at the isoelectric point is due to an ionization of the isoelectric protein by the trivalent or tetravalent ions.

4. This ionization might be due to a change of the pH of the solution, but experiments are reported which show that in addition to this influence on pH, $LaCl_3$ causes an ionization of the protein in some other way, possibly by the formation of a complex cation, gelatin-La. $Na_4Fe(CN)_6$ might probably cause the formation of a complex anion of the type gelatin-$Fe(CN)_6$. Isoelectric gelatin seems not to form such compounds with Ca, Na, Cl, or SO_4.

5. Solutions of $LaCl_3$ and $Na_4Fe(CN)_6$ influence the osmotic pressure of solutions of isoelectric gelatin in a similar way as they influence the P.D., inasmuch as in lower concentrations they raise the osmotic pressure of the gelatin solution until a maximum is reached at a concentration of about M/2,048 $LaCl_3$ and M/4,096 $Na_4Fe(CN)_6$. A further increase of the concentration of the salt depresses the osmotic pressure again. NaCl, LiCl, $MgCl_2$, $CaCl_2$, and Na_2SO_4 do not act in this way.

6. Solutions of $LaCl_3$ have only a depressing effect on the P.D. and osmotic pressure of gelatin chloride solutions of pH 3.0 and this depressing effect is quantitatively identical with that of solutions of $CaCl_2$ and NaCl of the same concentration of Cl.

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NIZING INFLUENCE OF SALTS WITH TRIVALENT AND TETRAVALENT IONS ON CRYSTALLINE EGG ALBUMIN AT THE ISOELECTRIC POINT.

BY JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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I.

INTRODUCTION.

Measurements of the membrane potentials between aqueous protein solutions or gels and surrounding water at equilibrium have yielded the result that salts with trivalent cations give isoelectric protein a positive charge while salts with tetravalent anions give it a negative charge.[1] On the basis of Donnan's theory of membrane potentials it was assumed that salts with trivalent cations, *e.g.* $LaCl_3$, form with isoelectric protein ionizable salts which result in the formation of positive protein-La ions and negative Cl ions; and that salts like $Na_4Fe(CN)_6$ form with isoelectric protein salts which result in the formation of negative protein-Fe $(CN)_6$ ions and positive Na ions.[2] In other words, salts with trivalent cations react with isoelectric protein like acids, and salts with tetravalent anions react with isoelectric protein like alkalies; with this difference, however, that the compounds of isoelectric gelatin with acids and bases are much more stable than those with the salts of trivalent cations or tetravalent anions. Salts with divalent ions like Na_2SO_4, $CaCl_2$, or salts with monovalent ions like NaCl, did not produce any measurable charge on isoelectric gelatin in aqueous solutions. Experiments on anomalous osmosis through gelatin-collodion membranes were in harmony with these results.[3]

[1] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 741.

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 165.

[3] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 463.

It seemed of interest to find out whether experiments on the stability of aqueous solutions and suspensions of proteins at the isoelectric point are also in harmony with the results of the direct measurements of the membrane potentials. The reason why experiments on stability and flocculation were selected was that it is often stated that the flocculation of colloids is influenced in an opposite sense by the two oppositely charged ions of a salt, the ion with the same sign of charge as the colloid increasing the stability, the salt ion with the opposite sign of charge to that of the colloidal particle diminishing the stability of the suspension.

If we wish to use observations on the influence of salts on the stability of protein solutions at the isoelectric point for conclusions concerning the influence of ions on the electrical charges of particles, we are confronted with the difficulty that the electrical charges of particles are not the only forces which keep proteins in solution. There are two different kinds of forces determining the stability of solutions or suspensions of proteins, namely; first, the attraction between the molecules of the protein and the solvent, and second, forces of electrostatic repulsion between micellæ. When the forces of attraction between molecules of the solvent and molecules of the solute (which may be secondary valency forces) are greater than the forces of attraction between the molecules of the solute for each other, the solution will be stable. This type of force acts in the general case of solutions of crystalloids.

When the forces of attraction between the molecules of solute and solvent are weak, the molecules of the solute upon colliding may adhere to each other and aggregates will be formed. This aggregate formation will lead to a flocculation or coagulation of the whole mass unless new forces originate in the small nascent aggregates (or micellæ) which prevent their coalescence into larger aggregates. These forces may be electrical charges whereby the nascent micellæ repel each other. The writer has investigated the origin of these charges in the case of protein micellæ and has found that they are due to the establishment of a Donnan equilibrium between particles and solution.[4] A membrane equilibrium between particles and

[4] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 120; *J. Gen. Physiol.*, 1920–21, iii, 667; 1921–22, iv, 351.

solution can, however, only be established when the particles contain protein ions.

There exists a criterion which seems to permit us to decide which one of the two types of forces is responsible for the stability of a solution or suspension. When the stability depends upon the repulsive forces due to a potential difference between micellæ and solution, comparatively low concentrations of solutions will be required for the precipitation of the protein, since comparatively low concentrations of salts (*e.g.* concentrations of M/8 or less) suffice for the annihilation of the P.D. When, however, the stability of a solution is not determined by the P.D. between micellæ and solution but by forces of residual valency between molecules of solute and solvent, much higher concentrations of salts are, as a rule, required for precipitation than are sufficient for the annihilation of a P.D. caused by the Donnan equilibrium. Moreover, the efficiency of a salt in annihilating the P.D. between a micella and a solution is the lower the higher the valency of that ion of the salt which has the opposite sign of charge to that of the micella.

II.

The Prevention of Heat Coagulation of Isoelectric Egg Albumin by Trivalent and Tetravalent Ions.

Isoelectric crystalline egg albumin is quite soluble in water as long as the temperature is low. 8 per cent solutions kept at 2°C. remained perfectly clear for more than a year—and they would probably have kept clear indefinitely. Since it requires very high concentrations of salts to cause a precipitation of isoelectric crystalline egg albumin from aqueous solution at ordinary temperature, we may assume that the forces determining the stability of solutions of isoelectric crystalline egg albumin at sufficiently low temperature are not the electrical charges of micellæ but the attraction between molecules of isoelectric albumin and molecules of water. When, however, the temperature of a 1 per cent solution of isoelectric crystalline egg albumin is raised to about 73°C. or above, crystalline egg albumin is flocculated. Through the rise in temperature a change occurs in the molecule of crystalline egg albumin, whereby the attraction of the molecules of

albumin for each other becomes greater than the attraction between the molecules of albumin and water.

If the albumin is practically non-ionized (as is the case at the isoelectric point) no Donnan equilibrium between the nascent micellæ and the surrounding solution can be established and no P.D. between the nascent micellæ and the solution can prevent the coalescence of the micellæ. When, however, part of the albumin is ionized, the molecules of albumin will also unite upon heating to form micellæ, but these micellæ will begin to repel each other as soon as they contain protein ions. For in this case the protein ions in the nascent micellæ will cause the establishment of a Donnan equilibrium between the micellæ and the solution, and the electrical charge produced thereby on the particles will prevent the further coalescence of the nascent micellæ. This charge will increase with the relative concentration of ionized protein contained in the micellæ. It is obvious that the average size of the micellæ will remain the smaller the greater the relative concentration of protein ions in solution; since the greater the relative concentration of ionized protein the smaller will be the average number of protein molecules which can form an aggregate without including protein ions. This argument is supported by the well known fact that when we add some acid or alkali to isoelectric albumin, the solution will become only opalescent on heating but heat precipitation of the albumin will no longer occur. A comparison of the effect of increasing concentrations of acid shows that the relative size of the micellæ will become the smaller the greater the relative mass of ionized protein. To demonstrate this, 10 cc. of an aqueous 0.2 per cent solution of almost isoelectric crystalline egg albumin and containing varying amounts of 0.1 N HCl were put into test-tubes, and these test-tubes were put into boiling water until the temperature of the albumin solution rose to 90°C. Then the test-tubes were allowed to cool at room temperature and the appearance of the solution was noticed. Table I gives the result.

When the 10 cc. contained 0.01 cc. of 0.1 N HCl the protein remained practically isoelectric (pH 4.8), practically no ionization was produced, and hence flocculation occurred upon heating.

TABLE I.

Influence of HCl on Heat Coagulation of Crystalline Egg Albumin in Aqueous Solution. 10 cc. of 0.2 Per Cent Albumin (Nearly Isoelectric), Containing Various Concentrations of 0.1 N HCl, Heated to 90°C.

cc. 0.1 N HCl in 10 cc. of 0.2 per cent albumin	0	0.01	0.02	0.03	0.04	0.05	0.1	0.2	0.4	0.8	1.6	3.2
Appearance of solution	Coagulated.		Very opaque.	Very opalescent.	Clear but slightly opalescent.	Very clear, like water.						

The addition of 0.02 cc. of 0.1 N HCl prevented coagulation but the solution was opaque showing that only when the micellæ were comparatively large did they assume electrical charges; owing to the fact that the concentration of albumin ions was small compared with that of non-ionized protein. These charges sufficed, however, to prevent the further coalescence of the large micellæ. When the solutions contained 0.03 cc. of 0.1 N HCl the relative concentration of ionized protein was increased and hence the micellæ remained smaller; the solution was no longer opaque but opalescent. With the addition of 0.04 cc. of 0.1 N HCl the solution became very transparent, showing only slight opalescence. With the increasing concentration of ionized protein the average number of molecules in a micella was considerably diminished, and this small size of the average micellæ manifested itself in the greater transparency of the solution. With a still greater concentration of HCl the average size of the micellæ diminished still further and the solution became as clear as water.

When, however, the concentration of HCl was increased beyond a certain limit, the P.D. between the micellæ and solution was diminished again on account of the depressing effect of the Cl ions demanded by Donnan's theory. When 100 cc. of 1 per cent solution of originally isoelectric albumin contained 30 cc. of N HCl, the protein coagulated at a temperature of 66°C. In this case all the protein was practically ionized but the P.D. between the micellæ and the liquid was nevertheless depressed to zero on acccount of the high concentration of Cl ions.

By measuring the concentration of salt required to precipitate crystalline egg albumin from a 1 per cent solution in water of pH 3.0 at a temperature of 70°C. we can show that the forces preventing heat coagulation in this case are the electrical charges of the micellæ, since the concentration of salt required to cause precipitation is of the order of M/8 or below, and since sulfates are more efficient than chlorides.

The fact that ionization of protein prevents heat coagulation of albumin can be used to find out whether other electrolytes than acids or alkalies are able to produce ionization of isoelectric egg

albumin. If other ions, like La, Ca, Na, SO_4, have such an effect on aqueous solutions of isoelectric albumin, it should show itself in the prevention of heat coagulation and in the optical appearance of the albumin solution after heating.

The experimental procedure was as follows: 7 cc. of water of pH 4.8 (this pH being the isoelectric point of crystalline egg albumin) were added to 2 cc. of 1 per cent solution of isoelectric crystalline egg albumin (of course, also of pH 4.8) and then 1 cc. of a salt solution containing different salts of different concentration, but always of pH 4.8, was added. The test-tubes containing the 10 cc. of the mixtures were put into boiling water until the liquid in the test-tubes reached a temperature of 90°C. and then the test-tubes were taken out of the water bath and allowed to cool at room temperature. Table II gives the appearance of the various mixtures after standing over night.

These experiments show first that the heat coagulation of isoelectric solutions of crystalline egg albumin is prevented by the addition of low concentrations of $LaCl_3$ or $Na_4Fe(CN)_6$ of pH 4.8. The concentration of $LaCl_3$ sufficient for this purpose was M/5,000 and that of $Na_4Fe(CN)_6$ about the same. Hence these two salts acted on the heat coagulation of isoelectric egg albumin like acids or alkali respectively. Moreover, it is obvious from Table II that at first the size of the micellæ formed diminishes with increasing concentration of $LaCl_3$ ions until the molecular concentration of $LaCl_3$ is about M/160. With a further increase of concentration of salt the size of the micellæ increases again (at 3 M/80) owing to the fact that the P.D. is depressed by the Cl ions; and at M/20 $LaCl_3$ this depressing action of the Cl ions on the P.D. is sufficient to permit again the heat coagulation of the albumin. In the case of $Na_4Fe(CN)_6$ the solution ceases to be clear when the concentration becomes 6M/80; in this case the depressing action of the Na ions on the P.D. of the negatively charged micellæ is so great that the micellæ begin to coalesce again.

None of the other salts tried, $CaCl_2$, $BaCl_2$, NaCl, or Na_2SO_4, is able to prevent heat coagulation of isoelectric egg albumin in an aqueous solution of pH 4.8. It is, of course, possible that certain

TABLE II.

Influence of Different Salts on Heat Coagulation of Crystalline Egg Albumin in Aqueous Solution at pH of Isoelectric Point.

Total concentration of salt in 10 cc. of 0.2 per cent albumin	10m/80	8m/80	6m/80	4m/80	3m/80	2m/80	m/80	m/160	m/320	m/640	m/1,280	m/2,560	m/5,120	m/10,240	m/20,480	m/40,960	m/81,920	0
$LaCl_3$	Coagulated.				Opalescent.	Bluish clear.		Clearest.					Opalescent and turbid.	Milky.	Coagulated.			
$Na_4Fe(CN)_6$		Very opaque.	Slight opalescence.	Clear.						Increasing opalescence. →			Milky.				Coagulated.	
$BaCl_2$			Coagulated.															
$CaCl_2$						Coagulated.												
Na_2SO_4						Coagulated.												
NaCl						Coagulated.												

other bivalent and monovalent ions act differently, since the valency is not the only variable determining the combining action.[5]

These results are in entire agreement with the experiments published in a preceding paper[1] showing that only salts with trivalent and tetravalent ions can produce a membrane potential on isoelectric gelatin while Na, Ca, Ba, and SO_4 have no such effect.

When crystalline egg albumin is dissolved in a solution with little water and much alcohol, salts have not the same influence on the stability of the solution that they have in an aqueous solution. This is due to the difference in the nature of the solvent, since the influence of salts on the stability of such alcoholic solutions of crystalline egg albumin is similar to the influence of salts on the stability of solutions of gelatin in a solution with much alcohol and little water. The stability of isoelectric gelatin in a mixture with little water and much alcohol is increased not only by salts with trivalent and tetravalent ions but also by salts with bivalent ions, such as $MgCl_2$, $CaCl_2$, $SrCl_2$, $BaCl_2$, and Na_2SO_4, while salts like $MgSO_4$, LiCl, NaCl, or KCl have no such effect. The clearing effect of Ba was considerably greater than that of Mg. We know too little about the P.D. and solubility in alcoholic solutions and for this reason the publication and discussion of these results may be postponed.

SUMMARY AND CONCLUSION.

1. While crystalline egg albumin is highly soluble in water at low temperature at the pH of its isoelectric point, it is coagulated by heating. It has long been known that this coagulation can be prevented by adding either acid or alkali, whereby the protein is ionized.

2. It is shown in this paper that salts with trivalent or tetravalent ions, *e.g.* $LaCl_3$ or $Na_4Fe(CN)_6$, are also able to prevent the heat coagulation of albumin at the isoelectric point (*i.e.* pH 4.8), while salts with a divalent ion, *e.g.* $CaCl_2$, $BaCl_2$, Na_2SO_4, or salts like NaCl, have no such effect.

[5] Michaelis, L., and Rona, P., *Biochem. Z.*, 1919, xciv, 225.

3. This is in harmony with the fact shown in a preceding paper that salts with trivalent or tetravalent ions can cause the ionization of proteins at its isoelectric point and thus give rise to a membrane potential between micellæ of isoelectric protein and surrounding aqueous solution, while the above mentioned salts with divalent and monovalent ions have apparently no such effect.

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ON THE INFLUENCE OF AGGREGATES ON THE MEMBRANE POTENTIALS AND THE OSMOTIC PRESSURE OF PROTEIN SOLUTIONS.

BY JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 25, 1922.)

I.

When a protein chloride solution is separated by a collodion membrane from a dilute HCl solution free from protein, under suitable experimental conditions an osmotic equilbrium is reached after about 6 hours. When at that time the hydrogen ion concentrations of the protein solution and the outside solution are measured, it is found that the hydrogen ion concentration of the outside solution is higher than that of the protein solution, while the chlorine ion concentration is higher in the protein solution than outside.[1] This unequal concentration of the oppositely charged H and Cl ions inside and outside leads to a membrane potential and modifies the osmotic pressure of the protein solution. The values of both effects can be calculated from the differences in the pH (or pCl) inside and outside on the basis of Donnan's equation for membrane equilibria. The agreement between observed and calculated values on the basis of Donnan's equilibrium equation is excellent for the membrane potentials and equally good for the osmotic pressure, except that a slight difference in pH (in the second decimal) has a much greater influence on the calculated values of osmotic pressure than of the P.D. These facts were based on the writer's observations on solutions of gelatin and crystalline egg albumin[1] and they were confirmed by Hitchcock's observations on solutions of edestin.[2]

The writer has shown that if in a solution of gelatin chloride of a certain pH, part of the gelatin in solution is replaced by the

[1] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922; *J. Gen. Physiol.*, 1920–21, iii, 667, 691.

[2] Hitchcock, D. I., *J. Gen. Physiol.*, 1921–22, iv, 597.

same weight of powdered gelatin (without changing the pH) the osmotic pressure of the solution is lowered, and this lowering of the osmotic pressure increases the more the more of the dissolved gelatin is replaced by powdered gelatin.[3] This shows that only that part of the protein which is in true solution, *i.e.* which does not exist in the form of larger aggregates, influences the osmotic pressure of a solution. The question arose whether or not powdered particles of gelatin would influence the membrane potential. To obtain an answer to this question the following experiment was made.

Powdered gelatin going through the meshes of sieve 30 but not through 60 was rendered isoelectric in the way previously described. Part of this isolectric gelatin was melted and the melted and powdered isoelectric gelatin were mixed. The total weight of isoelectric gelatin in 100 cc. solution was always the same, but the proportion of powdered to dissolved gelatin varied as indicated in Table I. Thus when the weight of the powdered gelatin was 0.5 gm., the weight of the dissolved gelatin was about 0.3 gm.; when the weight of the powdered gelatin was 0.2 gm., that of the dissolved was 0.6 gm., etc. 100 cc. of the mixture contained 8 cc. of 0.1 N HCl, and the pH of the gelatin solution (at the equilibrium condition to be described) was between 3.2 and 3.3. At this pH the osmotic pressure of a gelatin solution is nearly a maximum. A 1 per cent solution of gelatin chloride has an osmotic pressure of about 450 mm. water at pH 3.4. Only a small part of this osmotic pressure is due to the osmotic pressure of the protein particles themselves; the rest of the observed osmotic pressure of gelatin chloride solutions of pH 3.4 is due to the excess concentration of the crystalloidal ions inside the collodion bag (in which the osmotic pressure of the gelatin solution is measured) over that of the outside aqueous solution free from gelatin, and this quantity is determined by the Donnan equilibrium.[4]

Collodion bags of a content of about 50 cc. were filled with these suspensions and closed with rubber stoppers perforated with glass tubes serving as manometers to measure the osmotic pressure.[4]

[3] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 232; *J. Gen. Physiol.*, 1921–22, iv, 97.

[4] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 169: *J. Geh. Physiol.*, 1920–21, iii, 691.

The bags were put over night at 21°C. into beakers containing each 350 cc. of 0.001 N HCl in water. The next day the osmotic pressure was read, the P.D. between the gelatin chloride solution and the outside aqueous solution free from gelatin was measured (with a Compton electrometer and indifferent saturated KCl-calomel electrodes), and the pH inside and outside was determined with the hydrogen electrode. Table I gives the results of these observations.

TABLE I.

Influence of Substitution of Powdered for Dissolved Gelatin on Osmotic Pressure and P. D.

Powdered gelatin per 100 cc., *gm.*	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0
Dissolved gelatin per 100 cc., *gm.*	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
Osmotic pressure	85	132	181	230	268	310	342	406	398
pH inside	3.16	3.20	3.18	3.19	3.22	3.27	3.28	3.30	3.33
pH ontside	2.82	2.85	2.85	2.83	2.83	2.85	2.82	2.84	2.87
pH inside minus pH outside	0.34	0.35	0.33	0.36	0.39	0.42	0.46	0.46	0.46
P.D. calculate *millivolts*	20.0	20.5	19.0	21.0	22.5	24.5	26.5	26.5	26.5
Membrane potentials observed, *millivolts*	Between 23.0 and 18.0	Between 22.0 and 18.0.	23.0	21.0	22.5	25.0	26.0	26.5	27.0
	No constant reading.								

The table confirms the writer's former observation that the osmotic pressure of the gelatin solution diminishes the more the more of the dissolved gelatin is replaced by powdered gelatin. The latter obviously does not participate in the osmotic pressure.

The table shows furthermore that the P.D. observed at equilibrium between the gelatin solution and the outside aqueous solution varies much less than the osmotic pressure. It became necessary to ascertain whether or not this P.D. across the membrane which was measured with the aid of two indifferent electrodes (saturated KCl-calomel solution) was actually determined by the difference in the

hydrogen ion concentrations inside and outside, as we should expect if the membrane potentials are due to a membrane equilibrium. The pH inside and outside was therefore measured with the aid of the hydrogen electrode and the value 58 (pH inside minus pH outside) millivolts is called the calculated P.D. The reader will notice that the difference between the observed membrane potential (measured with indifferent electrodes) and the calculated P.D. is not more than 0.5 millivolt. This leaves no doubt that the observed P.D. is determined by the difference in the hydrogen ion concentration on the opposite sides of the collodion membrane and that this P.D. obeys Donnan's equilibrium equation.[5]

These facts then show that the protein aggregates participate in the Donnan equilibrium almost to the same extent as do the isolated molecules or ions of gelatin, and this participation finds expression in the fact that the membrane potentials are lowered comparatively little when dissolved gelatin is replaced by powdered gelatin. The same particles, however, do not contribute to the osmotic pressure for the reason that their share in the excess of chlorine ions is contained inside the solid particles, where it serves to increase the swelling of the particles. The swelling of solid protein particles is, as Procter and Wilson have shown,[6] due to the increase of osmotic pressure inside the particles caused by the Donnan equilibrium. In our experiment there exists inside of each particle of powdered gelatin a Donnan equilibrium whereby the concentration of Cl ions inside is greater than outside and this causes an osmotic pressure. Water will, therefore, diffuse into each granule until the cohesion pressure of the solid particles of gelatin equals the osmotic pressure inside the particles due to the Donnan equilibrium, and the particles will swell. When we therefore have a mixture of dissolved gelatin and powdered particles (micellæ) we have two different osmotic pressures; namely, first, the osmotic pressure of the gelatin in true solution, and, second, the osmotic pressure inside each solid particle of gelatin. The former is measured by the hydrostatic pressure of the column of water required to equalize the rate of dif-

[5] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 120; *J. Gen. Physiol.*, 1920–21, iii, 557, 667; 1921–22, iv, 351, 463.

[6] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

fusion in opposite directions through the membrane. This is the osmotic pressure of the protein solution in Table I. The osmotic pressure inside each particle of solid powdered gelatin results in swelling, *i.e.* in an increase of the force of cohesion between the molecules of the gel particle, and this effect does not appear in the osmotic pressure of the solution. Only that part of the osmotic forces in a protein solution appears in the form of hydrostatic pressure which is directly or indirectly due to the isolated molecules of the protein; and this hydrostatic pressure is diminished when part of the protein in solution is replaced by aggregates or micellæ of protein.

II.

When a solution of gelatin chloride containing solid granules of gelatin is separated by a collodion membrane from an aqueous solution (free from protein) two different equilibria are established; one across the membrane between the aqueous solution outside and the gelatin solution *inside* the membrane, and a second one between the solid granules of gelatin and the gelatin solution in which the granules are suspended. At first thought it might seem strange that when solid granules of isoelectric gelatin are suspended in a solution of gelatin and HCl, there should arise a difference in the distribution of the H and Cl ions inside the solid granules and the surrounding gelatin solution. Yet this is the case, as Table II shows, and the reason is easily understood. In the solid granules of gelatin the concentration of protein molecules is much higher than in the weak solutions of gelatin surrounding the granules, and if HCl is added the concentration of gelatin ions must be higher inside the solid gelatin granules than in the dilute gelatin solution in which the granules are suspended. It follows from Donnan's theory that this difference in the concentration of protein ions inside the powdered particles and the solution must give rise to a Donnan equilibrium; as a consequence of which a P.D. must exist between the solid particles and the weaker gelatin solution.[7]

This consequence of the theory was confirmed by the following experiment. Mixtures of a solution of isoelectric gelatin and

[7] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 145.

powdered isoelectric gelatin were made so that 100 cc. always contained 0.8 gm. of isoelectric gelatin in all. The proportion of solid and liquid gelatin varied, however, in each case as indicated in Table II. In each 100 cc. of the mixture were contained 8 cc. of 0.1 N HCl. The mixtures were kept for 2 hours at 20°C. and frequently agitated to accelerate establishment of equilibrium between granules and solution. The solid powdered gelatin was then separated from the supernatant liquid by filtration.

TABLE II.

Donnan Equilibrium between Particles of Powdered Gelatin and Gelatin in Solution.

Powdered gelatin per 100 cc., *gm*	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
Dissolved gelatin per 100 cc., *gm*	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0
pH of powdered gelatin		3.30	3.26	3.28	3.24	3.28	3.24	3.30	3.26
pH of supernatant gelatin solution	2.99	2.97	2.90	2.88	2.83	2.77	2.72	2.69	2.62
pH solid minus pH liquid gelatin		0.33	0.36	0.40	0.41	0.51	0.52	0.61	0.64
P.D. calculated, *millivolts*		19.0	21.0	23.0	24.0	29.5	30.0	35.5	37.0
P.D. observed, *millivolts*		14.5	17.0	17.0	17.5	23.0	26.0	30.0	33.0

The solid gelatin was melted and poured into the vessels described on page 154 of a recent book by the writer, and the P.D. between the solid and liquid gelatin was measured. The values are found in Table II, showing that there exists a considerable P.D. between the gelatin granules and weak solutions of gelatin, and that this P.D. increases with the relative increase in the concentration of solid gelatin, as was to be expected.

Measurements of the pH in the solid and liquid gelatin showed that this P.D. was determined by the Donnan equilibrium.[8] There exists, however, a discrepancy between calculated and observed values for the P.D., which requires further investigation. On the whole, however, the figures seem to prove that when a suspension of powdered protein in a weak gelatin solution is put inside a collodion bag, the latter dipped into an aqueous solution free from gelatin, two equilibria are established; namely, one between the solid

[8] The measurements of the P.D. between solid gelatin and solution are not as accurate as the measurements between liquids across a membrane.

gelatin and the solution of gelatin inside the collodion bag, and a second one between the gelatin solution inside the collodion bag and the outside aqueous solution free from gelatin.

This explains why the powdered particles of protein contained in a gelatin solution participate in the Donnan equilibrium and the membrane potential of the solution without adding to the osmotic pressure of the solution as measured by the hydrostatic pressure required to equalize the rate of diffusion of water in opposite directions through the membrane. The participation of the solid particles in the Donnan equilibrium leads to an osmotic pressure inside of each solid granule, but this osmotic pressure is measured in terms of cohesion pressure of the swollen particles.

This swelling of the solid particles increases the viscosity of the solution, and the writer has shown that this fact is a further support of the explanation of colloidal behavior on the basis of the theory of membrane equilibria.[3]

SUMMARY AND CONCLUSIONS.

1. It is shown that when part of the gelatin in a solution of gelatin chloride is replaced by particles of powdered gelatin (without change of pH) the membrane potential of the solution is influenced comparatively little.

2. A measurement of the hydrogen ion concentration of the gelatin chloride solution and the outside aqueous solution with which the gelatin solution is in osmotic equilibrium, shows that the membrane potential can be calculated from this difference of hydrogen ion concentration with an accuracy of half a millivolt. This proves that the membrane potential is due to the establishment of a membrane equilibrium and that the powdered particles participate in this membrane equilibrium.

3. It is shown that a Donnan equilibrium is established between powdered particles of gelatin chloride and not too strong a solution of gelatin chloride. This is due to the fact that the powdered gelatin particles may be considered as a solid solution of gelatin with a higher concentration than that of the weak gelatin solution in which they are suspended. It follows from the theory of membrane

equilibria that this difference in concentration of protein ions must give rise to potential differences between the solid particles and the weaker gelatin solution.

4. The writer had shown previously that when the gelatin in a solution of gelatin chloride is replaced by powdered gelatin (without a change in pH), the osmotic pressure of the solution is lowered the more the more dissolved gelatin is replaced by powdered gelatin. It is therefore obvious that the powdered particles of gelatin do not participate in the osmotic pressure of the solution in spite of the fact that they participate in the establishment of the Donnan equilibrium and in the membrane potentials.

5. This paradoxical phenomenon finds its explanation in the fact that as a consequence of the participation of each particle in the Donnan equilibrium, a special osmotic pressure is set up in each individual particle of powdered gelatin which leads to a swelling of that particle, and this osmotic pressure is measured by the increase in the cohesion pressure of the powdered particles required to balance the osmotic pressure inside each particle.

6. In a mixture of protein in solution and powdered protein (or protein micellæ) we have therefore two kinds of osmotic pressure, the hydrostatic pressure of the protein which is in true solution, and the cohesion pressure of the aggregates. Since only the former is noticeable in the hydrostatic pressure which serves as a measure of the osmotic pressure of a solution, it is clear why the osmotic pressure of a protein solution must be diminished when part of the protein in true solution is replaced by aggregates.

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THE INTERPRETATION OF THE INFLUENCE OF ACID ON THE OSMOTIC PRESSURE OF PROTEIN SOLUTIONS.

BY JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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I.

The osmotic pressure of protein solutions is influenced by electrolytes in the same way as are the other colloidal properties, such as viscosity, swelling, or membrane potentials, and this suggests that the influence of electrolytes on all these properties may be due to the same cause. In a series of papers[1] and a recent book[2] the writer has tried to show that this common cause is the establishment of Donnan's membrane equilibrium, which is produced when a protein solution containing ionized protein is separated by a collodion membrane from an aqueous solution free from protein; or when a gel containing protein ions is surrounded by an aqueous solution free from protein or containing little protein. When the collodion membrane or the gel is permeable to crystalloidal ions but impermeable to protein ions, an equilibrium condition originates in which the product of the concentrations of each pair of oppositely charged diffusible (crystalloidal) ions is the same on opposite sides of the membrane. This results in an unequal distribution of the different crystalloidal ions on the opposite sides of the membrane, whereby the osmotic pressure of the protein solution is modified. The observed osmotic pressure of a protein solution must therefore be corrected for the Donnan effect before it can be used for theories of the effect of electrolytes on the osmotic pressure of protein solutions. When 1 g. of iso-electric protein, gelatin or crystalline egg albumin or casein or edestin, is

[1] Loeb, *J. Gen. Physiol.*, **3**, (a) 667, (b) 691, (c) 827 (1920–1), (d) **4**, 73 (1921–2).

[2] Loeb, "Proteins and the Theory of Colloidal Behavior," McGraw-Hill Book Company, Inc., New York and London, 1922.

dissolved in 100 cc. of water containing varying quantities of 0.1 N hydrocholoric acid, protein chloride is formed. In this salt formation, the hydrogen ion becomes part of the complex protein cation, while the chlorine ion remains free as before. That the protein chloride formed is highly ionized is shown by the fact that the addition of iso-electric protein to a solution of hydrochloric acid lowers the concentration of hydrogen ion but not of chloride ion, as measured by the hydrogen and calomel electrodes, respectively,[3] indicating that the acid reacts with protein much as it does with ammonia. There is, however, one essential difference between the two cases, inasmuch as the protein chloride solutions are hydrolyzed to a considerable extent, so that there exists always an equilibrium between free hydrochloric acid, protein chloride and iso-electric protein not in combination with the acid. The relative part of acid in combination with protein is an unequivocal function of the hydrogen-ion concentration of the protein solution. Since only the ionized part of the protein plays a rôle in the Donnan equilibrium and since this part is determined by the hydrogen-ion concentration of the solution, a protein solution is only adequately defined if, aside from the concentration of originally iso-electric protein, the hydrogen-ion concentration of the solution is known.

When a solution of a protein chloride is put into a collodion bag, permeable to crystalloidal ions but not to the protein ions, and when this bag is put into water or a weak hydrochloric acid solution, there exist, when osmotic equilibrium is established, inside the collodion bag, free hydrochloric acid, protein chloride and non-combined (iso-electric) protein, while outside there is free hydrochloric acid. At equilibrium let x be the concentration of hydrogen and of chloride ions in the outside aqueous solution (free from protein), y the concentration of the hydrogen and chloride ions of the free acid inside the protein solution, and z the concentration of the chloride ions in combination with the protein ions, then according to Donnan the equilibrium is expressed by the following equation (which was first used by Procter and Wilson in their theory of swelling),[4]

$$x^2 = y(y + z) \qquad (1)$$

[3] Ref. 2, pp. 41–42.

[4] Procter, *J. Chem. Soc.*, **105**, 313 (1914). Procter and Wilson, *ibid.*, **109**, 307 (1916).

Since all the quantities in this equation are positive, x must be greater than y, and $y + z$ must be greater than x. It is also evident from Equation 1 that $2y + z$ is greater than $2x$, showing that the sum of the concentrations of hydrogen and chloride ions must be greater in the inside than in the outside solution at equilibrium. This fact is, as we shall see, of great importance for the understanding of the effect of acids on the osmotic pressure of protein solutions. The values of y and x can be determined directly with the aid of the hydrogen electrode or by titration, and z can be calculated from Equation 1. We can, therefore, calculate with the aid of Donnan's equilibrium equation the correction which the observed osmotic pressure of a protein chloride solution requires, provided that we can prove that the inequality of the concentrations of hydrogen and chloride ions on the opposite sides of the membrane is caused by the membrane equilibrium. This proof has been furnished through the comparison of the membrane potentials of protein chloride solutions with the values of these potentials calculated from differences of the hydrogen-ion or the chlorine-ion concentrations on the opposite sides of the membrane with the aid of Donnan's equilibrium formula. The two sets of values agree perfectly.[5]

When 1 g. of iso-electric crystalline egg albumin or of iso-electric gelatin[6] or of iso-electric edestin[7] is dissolved in 100 cc. of water containing various quantities of 0.1 N hydrochloric acid, and when the osmotic pressure of these solutions is measured after 18 hours it is found that the osmotic pressure rises at first with increasing concentration of the hydrogen ions until a maximum is reached (at about P_H 3.4) and then falls again with a further rise in the hydrogen-ion concentration. The dispersion theory explains this rise on the assumption that the addition of little acid increases the degree of dispersion of the solution thereby raising the osmotic pressure, while the addition of more acid diminishes the degree of dispersion again, thereby diminishing again the osmotic pressure of the solution.[8] This

[5] Ref. 1 (a); *J. Gen. Physiol.*, **4**, (a) 351, (b) 617, (c) 621 (1921–22). Ref. 2, p. 120.

[6] Ref. 2, p. 169. Ref. 1 (b).

[7] Hitchcock, *J. Gen. Physiol.*, **4**, 597 (1921–22).

[8] Zsigmondy, "Kolloidchemie," Otto Spamer, Leipzig, 2nd ed., 1918, p. 341.

is merely a qualitative speculation which at best does not conflict with the observations on the influence of acid on the osmotic pressure of protein solutions; it conflicts, however, with the observations on the influence of acids on viscosity. The viscosity of solutions of gelatin or casein is also a minimum at the iso-electric point, rising upon the addition of a little acid until a maximum is reached and diminishing upon the addition of more acid. Unfortunately for the dispersion hypothesis, the writer has been able to show by experiments on the viscosity of suspensions of powdered gelatin in water that the viscosity of the suspensions is diminished considerably when the degree of dispersion of the suspension is increased.[9] If the dispersion theory were right in ascribing the increase of the osmotic pressure upon the addition of little acid to an increase in the degree of dispersion of the protein, the same increase in dispersion should diminish the viscosity of the protein solution. In reality, however, the addition of little acid raises the viscosity of the protein solution in the same way as it raises the osmotic pressure.

There exists a second difficulty overlooked by the believers in the dispersion theory, namely, that at the time of osmotic equilibrium the hydrogen-ion concentrations are different on the opposite sides of the membrane. This demands, as already stated, a correction of the observed values for the osmotic pressure of the protein solutions. The writer has already shown in experiments on the influence of acids on the osmotic pressure of solutions of gelatin and crystalline egg albumin that the correction covers practically the whole influence of acids on the osmotic pressure of these protein solutions, so that when the correction is made there is—at least within the limits of the accuracy of the experiments and calculations—little if anything left for the dispersion theory to explain.[8] This has been confirmed by Hitchcock in experiments on the influence of acid on edestin.[7] What was believed to be the effect of the variation of dispersion of the protein turned out to be the effect of the unequal concentration of crystalloidal ions on the opposite sides of the membrane demanded by Donnan's theory.

[8] Ref. 1 (d), p. 97. Ref. 2, p. 232.

It is intended to show in this paper that the same explanation is true also for the influence of acids on the osmotic pressure of casein solutions.

II.

Since the method used in these experiments has already been described,[6] it may suffice here to state briefly that the protein solutions were put into collodion bags cast in the shape of Erlenmeyer flasks of a volume of about 50 cc. The opening of each collodion bag was closed by a rubber stopper which was perforated by a glass tube serving as a manometer. The bag was dipped into a beaker containing 350 cc. of water which was at the beginning of the experiment usually brought to the same hydrogen-ion concentration as that of the casein solution by adding the necessary quantity of acid. The temperature was kept constant at 24°. The osmotic equilibrium was usually established within 6 hours, but the final reading was taken after about 18 or 24 hours. The osmotic pressure was measured in terms of the height in millimeters of the solution in the tubes.

The material used was casein prepared from skimmed milk after the method of Van Slyke and Baker.[10] According to Michaelis, the iso-electric point of casein is at a Sörensen value of about 4.7 (equivalent to a hydrogen-ion concentration of $2 \times 10^{-5} N$). The finely powdered casein used by us was nearly iso-electric. Casein is only sparingly soluble in water at the iso-electric point but it becomes more soluble in hydrochloric or phosphoric acid if enough of the acid is added. Portions of 1 g. of powdered casein were put into 100 cc. of water containing various quantities of 0.1 N hydrochloric acid (see Table I). After 24 hours all the casein was dissolved in those solutions which contained more than 5 and less than 40 cc. of 0.1 N hydrochloric acid in 100 cc. The hydrogen-ion concentration of the casein chloride solutions was determined after the casein was dissolved and each collodion bag containing a casein chloride solution was dipped into a beaker containing 350 cc. of hydrochloric acid of originally the same hydrogen-ion concentration as that of the casein solution.

The first row in Table I gives the volume of 0.1 N hydrochloric acid

[10] Van Slyke and Baker, *J. Biol. Chem.*, **35**, 127 (1918).

TABLE I.

Difference of Hydrogen-ion Concentration between Casein Solution and Outside Solution at Equilibrium.

1. Cc. of 0.1 *N* HCl contained in 100 cc. of solution....	1	2	3	4	5	6	7	8	10	12.5	15	17.5	20	25	30	40
2. Appearance of solution.....	Precipitate				Slight ppt.	Solution										Precipitate
	P_H at beginning of experiment															
3. P_H of casein (inside) solution...	3.73	3.43	3.30	3.20	3.10	2.94	2.78	2.64	2.45	2.26	2.13	2.02	1.93	1.81	1.71	1.55
4. P_H of outside solution.....	3.90	3.80	3.50	3.40	3.20	3.00	2.90	2.60	2.50	2.30	2.20	2.10	1.95	1.80	1.70	1.60
	P_H after 18 hours (at equilibrium)															
5. P_H of casein (inside) solution...	4.04	3.87	3.68	3.61	3.46	3.32	3.22	2.93	2.78	2.62	2.52	2.39	2.22	2.04	1.89	1.73
6. P_H of outside solution.....	3.84	3.69	3.42	3.28	3.13	2.97	2.88	2.67	2.57	2.42	2.35	2.26	2.14	2.00	1.88	1.73

TABLE II.

Approximate Agreement between Observed Osmotic Pressure and Osmotic Pressure Calculated from Donnan's Equation.

1. P_H of casein solution at equilibrium.....	1.73	1.89	2.04	2.22	2.39	2.52	2.62	2.78	2.93	3.22	3.32	3.46	3.61	3.68	3.87	4.04
2. $C_H{}^+ \times 10^5$ inside (y).....	1862	1288	912	603	407	302	240	166	118	60.3	47.9	34.7	24.6	20.9	13.5	9.1
3. $C_H{}^+ \times 10^5$ outside (x).....	1862	1318	1000	724	550	447	380	269	214	132	107	74.1	52.5	38.0	20.4	14.5
4. $\frac{(x+y)\ (x-y)}{y} = z$..	0	61	185	267	337	360	362	270	270	229	191	124	87.5	48.1	17.3	14.0
5. $2y + z - 2x$....	0	1	9	25	51	70	82	64	78	86	73	45	32	14	3.5	3.2
6. Share of osmotic pressure due to Donnan equilibrium, mm. of water.....	0	2.5	22.5	62.5	128	175	205	160	195	215	183	113	80	35	8.7	8
7. Observed osmotic pressure, mm. of solution.....	41	69	85	102	126	145	158	177	187	189	173	135	77	43	23	14

originally contained in 100 cc. of solution with 1 g. of originally approximately iso-electric casein. The next row states whether or not all the casein went into solution in the next 24 hours, that is, whether or not there was a precipitate at the bottom of the beaker containing the solution. It is evident that no more precipitate was left when the solution contained more than 5 and less than 40 cc. of 0.1 *N* hydrochloric acid in 100 cc. The third row gives the Sörensen value (P_H) of the casein solutions at the beginning of the osmotic pressure experiment. The next row gives the Sörensen value of the outside solution at the beginning. It was approximately identical in each case with that of the inside solution.

The important part of the table is the last two rows (5 and 6) giving the Sörensen values of the inside and outside solutions *after osmotic equilibrium was established*, that is, after 18 hours. The Sörensen value has changed both in the inside and outside solutions, but it is without exception smaller in the outside than in the inside solution; or in other words, the outside solution has at equilibrium a higher hydrogen-ion concentration than the inside solution. This is exactly what should happen according to Donnan's formula for membrane equilibria as stated above.

In Row 7, Table II, are given the observed values of osmotic pressure and the Sörensen value of the casein solutions as found at the end of the experiment after equilibrium was established is given in Row 1 of the same table. It is obvious that the osmotic pressure is a minimum nearest the iso-electric point (P_H 4.04), that it rises with the increase of acid until the maximal osmotic pressure is reached at about P_H 3.0, and that the osmotic pressure falls again when the hydrogen-ion concentration in the solution increases beyond this point. This is typical for the influence of acids on all colloidal properties of proteins and the question arises: What is the cause of this peculiar influence of the hydrogen-ion concentration? It has already been stated that the dispersion hypothesis explains this phenomenon by assuming that the addition of little acid increases the degree of dispersion and hence the osmotic pressure of the solution; while when more acid is added the degree of dispersion and hence the osmotic pressure are diminished again. Even if we were willing to ignore the observations on viscosity, we must consider the fact expressed in Rows 5 and 6 of Table I

(and demonstrated for other proteins than casein) that at equilibrium the hydrogen-ion concentration is not the same inside and outside the protein solution. This fact proves that the observed osmotic pressure of a protein chloride solution is the sum of two osmotic pressures, namely, that due to the concentration of the protein molecules, ions, and micells themselves, and that due to the excess of the concentration of crystalloidal ions (in our experiment hydrogen and chloride) inside the protein solution over the concentration of hydrogen and chloride ions in the outside solution. Before entering upon speculations concerning the possible cause of the influence of acid on the osmotic pressure of a casein chloride solution we must, therefore, find out how much of the influence of the acid is due to that part of the osmotic pressure which is the mere consequence of the excess of the concentration of hydrogen and chloride ions inside over that outside. This calculation is made in Table II, and it turns out that the effect of the hydrogen-ion concentration of the casein solution on its osmotic pressure is practically if not entirely due to the Donnan effect, that is, to the difference of the sum of the concentrations of hydrogen and chloride ions inside over the sum of the concentrations of these two ions outside.

At equilibrium let x be the concentration of free hydrogen and chloride ions in the outside solution and y the concentration of the hydrogen and chloride ions of the free hydrochloric acid in the inside (casein) solution; let z be the concentration of chloride ions in combination with the casein, and let us assume complete electrolytic dissociation of all the electrolytes. In that case the osmotic pressure due to the excess of concentration of hydrogen and chloride ions inside the casein solution over that in the outside solution is determined by the term, $2y + z - 2x$.

We can calculate the value of y from the measurement of the Sörensen value inside, and the value of x from the Sörensen value outside; the Sörensen value being the logarithm of the hydrogen-ion concentration with the minus sign omitted. We can calculate z from y and x with the aid of Equation 1 for the Donnan equilibrium,

$$z = \frac{(x+y)(z-y)}{y}$$

if we can furnish the proof that the excess of the concentration of crystalloidal ions inside over that outside is due to the Donnan equilibrium. This proof has been furnished for solutions of gelatin, crystalline egg albumin,[5] and edestin,[7] and we shall furnish it later for casein chloride solutions.

The calculation of $2y + z - 2x$ is carried out in Table II. Here the upper row is a repetition of the Sörensen values of the casein solutions at the end of the experiment from Row 5 of Table I. The second and third rows in Table II give the concentrations of hydrogen and chloride ions of the free acid inside and outside. The values of the concentration of hydrogen ion, C_{H^+}, are multiplied by 10^5. The fifth row gives the values of $2y + z - 2x$. These latter values are the excess of the concentration of hydrogen and chloride ions inside the casein solutions over the concentration of the same ions outside, on the assumption that the difference in P_H inside and outside is due to a Donnan equilibrium.

If we express the theoretical osmotic pressure of a gram molecular solution in terms of millimeters pressure of a column of water we have (with correction for a temperature of 24°)

$$22.4 \times 760 \times 13.6 \times \frac{297}{273} = 2.5 \times 10^5$$

In other words, a theoretical pressure of 2.5 mm. of water corresponds to a concentration of $10^{-5} N$. Hence, we need only multiply the values for $2y + z - 2x$ in Row 5 of Table II by 2.5 to obtain that part of the observed osmotic pressure of our casein solutions which is due to the Donnan effect. These values are given in the sixth row of Table II. When these values are compared with the observed osmotic pressures in the seventh row of Table II it is found that, within the limits of the accuracy of the measurements of Sörensen values and the calculation of z, there is a fair agreement. The observed osmotic pressures should be slightly higher than those due to the excess osmotic pressure of $2y + z - 2x$, namely, by that value which is due to the osmotic pressure of the protein itself. From the experiments with gelatin and crystalline egg albumin, the writer would infer that that share of the osmotic pressure which in this experiment was due to the casein molecules, ions, or aggregates was so small that it was within the limit of error of measurement and calculation.

In comparing the observed and calculated values for osmotic pressure in Table II the discrepancies appear to be rather large. This is simply due to the fact that a slight variation of the measurements of the Sörensen values in the second decimal place (so slight that it is inside the limit of the source of error) causes a considerable variation in the calculated value for $2y + z - 2x$. It is therefore worth while to point out that on the basis of the Donnan equation we can also calculate the Sörensen values inside or outside, and that in this case the calculated and the observed values show excellent agreement. This calculation was made by Dr. D. I. Hitchcock from the values in Table II, and I quote his results in his own words.

"It is possible to show in another way that the osmotic pressure of the casein chloride solutions is due almost entirely to the Donnan equilibrium. The expression for the difference in ion concentrations due to the Donnan equilibrium, $2y + z - 2x$, can be given in terms of x and y alone by substituting the value for z × from Equation 1.

$$2y+z-2x = 2y+\frac{x^2-y^2}{y}-2x = \frac{2y^2+x^2-y^2-2xy}{y} = \frac{x^2-2xy+y^2}{y} = \frac{(x-y)^2}{y}$$

(This expression was obtained by Procter and Wilson.[4])

"If it be assumed for the moment that the observed osmotic pressure is the result of the Donnan equilibrium alone, the correctness of this assumption can be tested by using the observed values for osmotic pressure and one hydrogen-ion concentration in the above expression, and solving for the other hydrogen-ion concentration.

"Let p = observed osmotic pressure in mm. of water. Then

$$p = 2.5 \times 10^5 \times \frac{(x-y)^2}{y}$$

if x and y are expressed in mols per liter.

$$(x-y)^2 = \frac{py}{250{,}000}; \quad x-y = \sqrt{\frac{py}{250{,}000}}; \quad x = y+\sqrt{\frac{py}{250{,}000}}; \quad x = y+\frac{\sqrt{py}}{500}$$

Using the values for y obtained from the measurement of the P_H inside, and the observed values for the osmotic pressure (Table II), calculated values for x were obtained. These were translated into terms of P_H (P_H outside = $-\log x$), and are given in Table III, together with the observed values for the P_H outside.

"The results show that in 10 out of 16 cases the agreement is within 0.02 P_H, which is about the experimental error of a single measurement of the P_H. If the other measurements of P_H were in error by that much in the opposite direction,

TABLE III

Comparison of Observed Values for P_H Outside with Those Calculated on the Assumption that the Osmotic Pressure Observed Was Due Entirely to the Donnan Equilibrium.

Observed P_H	1.73	1.88	2.00	2.14	2.26	2.35	2.42	2.57
Calculated P_H	1.69	1.83	1.96	2.12	2.26	2.36	2.44	2.56
Observed P_H	2.67	2.88	2.97	3.13	3.28	3.42	3.69	3.84
Calculated P_H	2.67	2.84	2.98	3.11	3.28	3.40	3.61	3.79

the agreement could be considered to be within the experimental error in all except one case. The calculation therefore shows quite conclusively that the observed osmotic pressure is wholly due, at least within the limits of the experimental error, to the differences in concentration of the crystalloidal ions which result from the Donnan equilibrium."

III.

We will now furnish the proof that (aside from this agreement between calculated and observed values of osmotic pressures) we had the right to assume that we are dealing here with the Donnan effect. The difference in the hydrogen-ion concentration inside (y) and outside (x) (Rows 2 and 3 in Table II) was not assumed but directly observed. What was assumed was the legitimacy of the calculation of z from Donnan's equilibrium Equation 1. In order to prove that this was justified we must be able to show that the value P_H inside minus P_H outside is exactly that demanded by Donnan's theory. This proof can be furnished by the measurements of the membrane potentials. There exists at osmotic equilibrium a potential difference between a protein chloride solution inside a collodion membrane and the protein-free hydrochloric acid solution outside, and this potential difference can be measured with the aid of indifferent saturated potassium chloride calomel electrodes. Donnan had shown that such a potential difference was to be expected on the basis of his theory.[11]

It follows from Donnan's formula that $E = \frac{RT}{F} \log \frac{x}{y} = \frac{RT}{F} \log \frac{y+z}{x}$.

If the difference between the Sörensen value inside and that out-

[11] Donnan, *Z. Elektrochem.*, **17**, 572 (1911).

side in Rows 5 and 6 of Table I is the consequence of the Donnan equilibrium, the potential difference across the collodion membrane between the casein chloride solution and the outside solution observed with indifferent calomel electrodes should be equal to the potential difference in millivolts calculated from either of the following two terms (at 24°): 59 (P_H inside minus P_H outside), or 59 (P_{Cl} outside minus P_{Cl} inside), regardless of whether the Sörensen value for hydrogen or chlorine is determined by titration or with a hydrogen electrode or a chloride electrode, respectively. The writer has shown that this is true in the case of gelatin and albumin chloride solutions,[5] and Hitchcock has shown that it is true for edestin solutions.[7] It is also true for casein chloride solutions as the following experiment shows: 4, 3, 2, 1, 0.5, and 0.25% solutions of iso-electric casein were brought to about P_H 2.5 by adding hydrochloric acid, put into collodion bags as described, and each bag was immersed in 350 cc. of hydrochloric acid solution of initial P_H 2.3. After 18 hours the potential differences between the casein solution (inside solution) and the outside aqueous solution free from casein were determined with the indifferent calomel electrodes and afterwards the Sörensen values of the inside and outside solutions were measured with the hydrogen electrode. Table IV gives the results of the measurements.

TABLE IV.

Agreement between Membrane Potentials and the Value 59 (P_H *Inside Minus* P_H *Outside*).

1. Casein chloride, %	4	3	2	1	0.5	0.25
2. P_H of inside solution at equilibrium.	2.595	2.595	2.580	2.53	2.46	2.46
3. P_H of outside solution at equilibrium	2.230	2.270	2.305	2.34	2.36	2.39
4. P_H inside minus P_H outside	0.365	0.325	0.275	0.19	0.10	0.07
5. 59 (P_H inside minus P_H outside)	21.5	19.2	16.2	11.2	5.9	4.1
6. P. d. observed with indifferent electrodes	20.0	18.0	15.0	10.8	7.2	3.1

The second and third rows give the Sörensen values of the inside and outside solutions as measured with the hydrogen electrode after osmotic equilibrium was established. The fourth row gives the values P_H inside minus P_H outside and the fifth row these values mul-

tiplied by 59, since the experiments were made at 24°. These latter values should agree with the values for observed potential differences between inside and outside solutions obtained with the aid of indifferent electrodes, and a comparison of the fifth and sixth rows of Table IV shows that the agreement is good. The agreement between the values in the last two rows of Table IV leaves little doubt that the difference, P_H inside minus P_H outside, is indeed the result of Donnan's membrane equilibrium. This, then, proves that our calculation of z was justified and that in order to obtain the influence of the hydrogen-ion concentration on the osmotic pressure of the protein particles themselves a deduction determined by $2y + z - 2x$ has to be made as we assumed. Of course, we may not have been justified in assuming complete dissociation of all the electrolytes involved, but this would necessitate only a minor correction. The main fact is that if the correction due to the Donnan effect is applied to the osmotic pressure of the casein chloride solution, it is found that the typical influence of the hydrogen-ion concentration upon the osmotic pressure of casein chloride solutions is practically accounted for by the Donnan effect. In this peculiar effect the protein plays only an indirect rôle, namely, that on account of the impermeability of the collodion membrane to protein ions the concentration of the diffusible crystalloidal ions becomes higher inside than outside. It is quite possible or probable that the acid also influences the degree of dispersion of the casein solution, but the influence of such an effect on the osmotic pressure of the protein solution is too small to be noticeable in our experiments.

SUMMARY AND CONCLUSIONS.

1. It is shown that the addition of a little acid to a solution of isoelectric casein increases the osmotic pressure until a maximum is reached after which the addition of still more acid depresses the osmotic pressure. This is explained on the basis of the dispersion hypothesis by the assumption that the addition of little acid increases the degree of dispersion and consequently the osmotic pressure of the protein solution, while the addition of more acid diminishes the degree of dispersion and consequently the osmotic pressure.

2. Our observations show that when osmotic equilibrium is estab-

lished between a solution of casein chloride enclosed in a collodion bag and an outside aqueous solution free from protein, the hydrogen-ion concentration is always greater in the outside solution than in the casein solution.

3. There exists a membrane potential between the casein chloride solution enclosed in a collodion bag and the surrounding aqueous solution free from protein with which the casein solution is in osmotic equilibrium, and this membrane potential can be measured with indifferent calomel electrodes and a Compton electrometer. When this is done at 24°, it is found that the number of millivolts of the observed membrane potential is equal to 59 (P_H inside minus P_H outside), the latter values being measured with the hydrogen electrode. This is the result to be expected if the inequality of the hydrogen-ion concentrations inside and outside the casein chloride solution at equilibrium is determined by Donnan's equation for membrane equilibria.

4. The fact that the hydrogen-ion concentration inside a protein chloride solution is not the same as that of the outside solution free from protein with which it is in osmotic equilibrium, shows that the observed osmotic pressure of the protein chloride solution cannot be entirely due to the protein but must be partly due to the difference in the concentration of the crystalloidal ions (hydrogen and chloride) in the inside and outside solution. It is, therefore, necessary to correct the observed osmotic pressure of a protein chloride solution for this difference in the concentration of hydrogen and chloride ions on the opposite sides of the membrane on the basis of the Donnan equilibrium. It is shown in this paper how this correction can be evaluated with the aid of Donnan's equation.

5. When this evaluation is made, it is found that within the limits of accuracy of the observations and calculations the entire effect of the hydrogen-ion concentration on the osmotic pressure of the casein chloride solution is covered by the correction required and that there is little if anything left for the dispersion hypothesis to explain.

6. This is in harmony with the conclusion previously reached by the writer that the influence of electrolytes on the osmotic pressure of protein solutions is entirely or almost entirely the consequence of the

difference in the concentration of crystalloidal ions inside the protein solutions and the outside aqueous solutions at equilibrium, this difference being caused by the establishment of a membrane equilibrium.

The writer is indebted to the editorial board for some valuable suggestions which have been incorporated in the text.

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THE COLLOIDAL BEHAVIOR OF SERUM GLOBULIN.

BY DAVID I. HITCHCOCK.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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I.

INTRODUCTION.

The theory of the colloidal behavior of proteins developed by Loeb[1] has been shown to apply to the proteins, gelatin, casein, egg albumin, and edestin.[2] It may be recalled that this theory is based on the idea that proteins are amphoteric electrolytes, reacting stoichiometrically with acids and bases to form ionizable salts, and on Donnan's theory of membrane equilibrium.[3] The object of the present work was to find out whether the theory would explain the behavior of a serum globulin as well.

The globulin was prepared from serum which was obtained by whipping and centrifuging fresh ox blood. The serum was diluted and the globulin was precipitated near its isoelectric point by CO_2 and purified as described by Robertson.[4] The product was therefore the insoluble serum globulin or euglobulin. The isoelectric point of this globulin was found by Rona and Michaelis[5] to be at a hydrogen ion concentration of 0.36×10^{-5} (pH 5.44). 1 per cent suspensions of the present preparation in distilled water were found to have a pH of 5.41 or 5.42 at 33°C. The removal of salts was assured by a measurement of the conductivity of a 1.628 per cent suspension

[1] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922; *J. Gen. Physiol.*, 1918–22, i–iv.

[2] Hitchcock, D. I., *J. Gen. Physiol.*, 1921–22, iv, 597.

[3] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572.

[4] Robertson, T. B., The physical chemistry of the proteins, New York, London, Bombay, Calcutta, and Madras, 1918, 40.

[5] Rona, P., and Michaelis, L., *Biochem. Z.*, 1910, xxviii, 193. Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914, 56.

of the globulin in distilled water; the specific conductivity at 34° was found to be 1.2×10^{-6} reciprocal ohms. The globulin was kept in suspension in distilled water saturated with thymol, and was preserved in an ice box. To obtain the concentrations of globulin required for the experiments, the bottle was shaken and samples were withdrawn by a pipette. The accuracy of this method of measuring the globulin was checked by dry weight determinations on two 25 cc. samples; each was found to contain 0.407 gm. of dry globulin.

Inasmuch as this globulin preparation did not give clear solutions with either acid or alkali, it is probable that it had become partly denatured or changed in some way during the process of purification. A second lot was prepared which gave nearly clear solutions in acid or alkali. Nevertheless, the first preparation could still be used to show whether or not a protein prepared from ox serum obeyed the same laws as other proteins.

II.

Titration of Globulin with Acids and Bases.

Titration curves were obtained by measuring with the hydrogen electrode, at 33°, the pH of 1 per cent solutions of the globulin in HCl and H_3PO_4 of various concentrations, and of a 0.5 per cent solution of the globulin in H_3PO_4. The pH values were referred to 0.1 M HCl, its pH being taken as 1.036. The results are given in Table I.

In order to determine how much of the HCl was combined with the globulin, the amounts of HCl required to give the same pH to 100 cc. of water, without protein, were subtracted from the total amounts of HCl in Table I. The figures for the acid-water curve have been given in a previous paper.[6] In the case of H_3PO_4 it was pointed out in connection with the titration of edestin[2] that this method does not give the true amounts of combined acid, on account of the repression of the ionization of the weak acid H_3PO_4 by the $H_2PO_4^-$ ion from the protein phosphate. Accordingly the amounts

[6] Hitchcock, D. I., *J. Gen. Physiol.*, 1921–22, iv, 733.

TABLE I.

Titration of Globulin with Acids.

0.1M HCl in 100 cc. of 1 per cent globulin, *cc.*	0	0	0.60	0.60	1.20	1.20	2.39	2,51	4.18	4.54	6.57	6.57	8.95	8.97	11.35	11.36	16.14	16.95	22.12	29.85	37.52	61.3
pH	5.41	5.42	3.99	4.07	3.71	3.80	3.43	3.39	3.16	3.07	2.85	2.86	2.60	2.62	2.35	2.36	2.04	2.08	1.85	1.67	1.52	1.29
0.1M H_3PO_4 in 100 cc. of 1 per cent globulin, *cc.*	1.39	2.78	5.56	9.72	16.7	24.3	34.0	43.6														
pH	3.63	3.33	2.98	2.65	2.33	2.14	1.98	1.88														
0.1M H_3PO_4 in 100 cc. of 0.5 per cent globulin, *cc.*	0.69	1.39	2.78	4.16	6.94	11.11	18.05	27.8	41.7	69.4												
pH	3.67	3.41	3.12	2.89	2.60	2.37	2.15	1.99	1.84	1.67												

of combined H_3PO_4 were calculated by the equation[7] which was used in the case of edestin

$$x = \frac{kc}{h+k} - h$$

Here x = concentration of $H_2PO_4^-$ from protein phosphate (assumed to be completely ionized).

k = primary ionization constant of H_3PO_4 = 0.01.

c = total concentration of H_3PO_4.

h = concentration of H^+ = concentration of H_2PO_4 from H_3PO_4.

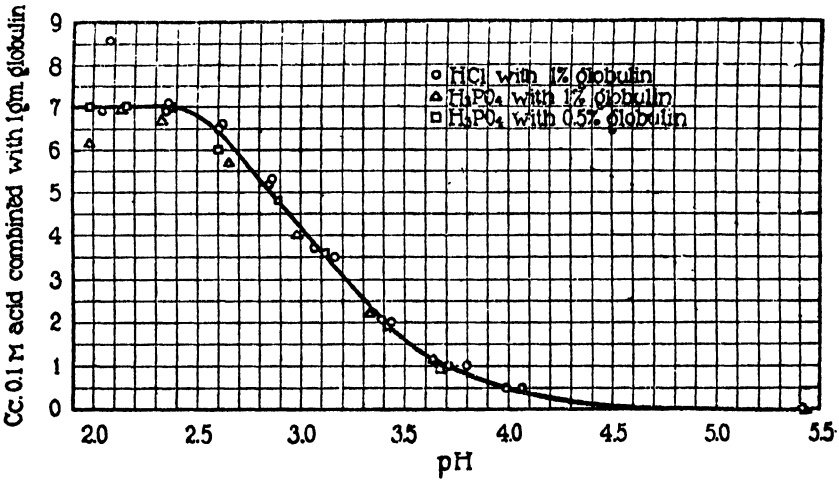

FIG. 1. Combination of globulin with acids.

The values obtained in this way, together with the values for HCl, are plotted in Fig. 1 in terms of cc. of 0.1 M acid combined with 1 gm. of globulin. Since the points fall on one curve it is to be inferred

[7] An equation identical with this was used by Pauli and Hirschfeld (Pauli, W., and Hirschfeld, M., *Biochem. Z.*, 1914, lxii, 245; Pauli, W., Kolloidchemie der Eiweisskörper, Dresden and Leipsic, 1920, pt. 1, 57) to calculate the amounts of acetic acid combined with horse serum albumin. However, since they plotted the amounts of combined acid against the total concentration of acid added instead of against the pH, they were not able to show that the protein was combined with chemically equivalent amounts of weak and strong acids.

that the globulin reacts stoichiometrically with equal numbers of molecules of the two acids. In other words, both HCl and H_3PO_4 react with globulin as monobasic acids. The combination curve appears to become horizontal at about 7 cc. of 0.1 M acid, indicating a combining weight of about 1,400 for the globulin. However, the height of the maximum is more or less uncertain. In attempting to carry the curve into the region of lower pH, it was found that the points for HCl varied irregularly on both sides of the value 7 cc.,

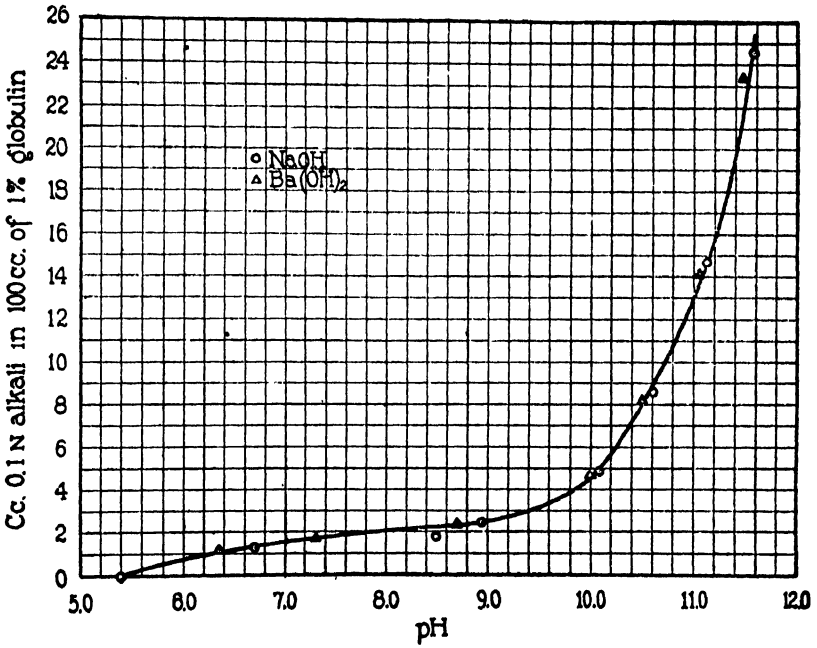

FIG. 2. Titration of globulin with alkalies.

while those for H_3PO_4 seemed to descend toward the axis of abscissæ. The former deviations are evidently due to the magnification of errors involved in taking the differences between two steep curves; the apparent decrease in the amount of H_3PO_4 combined is probably due to incomplete ionization of the globulin phosphate in the presence of much H_3PO_4, while in the calculation it was assumed that the protein phosphate was completely ionized. At any rate the results in Fig. 1 show that between pH 2 and 4 the globulin reacts stoichiometrically with equimolecular amounts of the two acids.

It should be added that a potentiometric titration of serum globulin with HCl was made by Robertson.[8] He calculated the amount of combined HCl simply by taking the difference between the total acid concentration present and the hydrogen ion concentration found in the presence of the protein. This involves the assumption of complete ionization of the HCl, which is probably correct for the concentrations he used, 0.01 M or below. His values fall at points higher than those in Fig. 1, and do not appear to reach a maximum in the range of acidity which they cover. The cause for this difference may lie in differences in the globulin preparations, or possibly in differences in temperature or in the standards of hydrogen ion concentration.

Fig. 2 represents the titration of 1 per cent globulin with NaOH and $Ba(OH)_2$. The curve is the original titration curve; no attempt was made to calculate the amount of combined alkali. These two strong alkalies appear to give the same titration curve with globulin when the concentrations are plotted in terms of normality, indicating that the globulin combines with them in equivalent, not in molecular proportions.

These titration experiments indicate that serum globulin behaves like gelatin, casein, egg albumin,[1] and edestin[2] in its stoichiometric reactions with acids and bases.

III.

Membrane Potentials.

In order to determine whether the Donnan equilibrium applied to the behavior of serum globulin, experiments were carried out by the method which had been used by Loeb[1] with gelatin and egg albumin and followed by the present writer[2] with edestin. Solutions were prepared containing 1 gm. of globulin in 100 cc. of HCl of various concentrations. These were placed in 50 cc. collodion bags fitted with rubber stoppers and manometer tubes, and the bags were suspended in beakers of HCl free from protein but of about the same pH as the protein solutions. The beakers were placed in a water bath at 25° ± 1°C., and about 20 to 24 hours were allowed for the

[8] Robertson, T. B., *J. Phys. Chem.*, 1907, xi, 437; The physical chemistry of the proteins, New York, London, Bombay, Calcutta, and Madras, 1918, 99.

attainment of equilibrium. Then the osmotic pressure was measured in terms of millimeters of the solution in the manometer tubes, and the P.D. between the inside and outside solutions was measured with the aid of saturated KCl-calomel electrodes and a Compton electrometer. The P.D. measurements were made at about 25°. The pH of the inside and outside solutions, at 33°, was then measured with the hydrogen electrode and potentiometer. The calculated P.D. values

TABLE II.

Effect of pH on P.D. *and Osmotic Pressure of 1 Per Cent Globulin Chloride.*

pH inside.....	4.57	4.38	3.86	3.55	3.35	3.06	2.67	2.19	1.72	1.28
pH outside.....	4.27	4.12	3.54	3.21	3.02	2.75	2.44	2.07	1.67	1.28
Observed P. D., *millivolts* .	6 5	7.0	16.5	21.0	21.0	20.0	15.0	10.5	4.0	3.0
Calculated P.D., *millivolts*.	17.5	15.0	19.0	20.0	20.0	18.0	13.5	7.5	3.0	0
Osmotic pressure, *mm.*...	9	12	23	38	57	73	71	48	27	45

Effect of pH on P.D. *and Osmotic Pressure of 0.5 Per Cent Globulin Phosphate.*

pH inside.....	3.57	3.26	2.95	2.76	2.50	2.30	2.09	1.97	1.83	1.66
pH outside.....	3.32	3.00	2.70	2.54	2.35	2.19	2.02	1.90	1.78	1.64
Observed P.D., *millivolts* .	12.0	13.0	13.0	11.5	9.0	7.0	5.0	4.0	3.5	2.5
Calculated P.D., *millivolts*.	15.0	16.0	14.5	13.0	8.5	7.0	4.5	4.0	3.0	1.5
Osmotic pressure, *mm.* ..	9	16	29	33	25	22	19	18	16	14

Effect of pH on P.D. *and Osmotic Pressure of 1 Per Cent Globulin Acetate in 0.01* M *Sodium Acetate.*

pH inside.....	4.63	4.34	4.02	3.69	3.35					
pH outside.....	4.61	4.31	4.01	3.63	3.31					
Observed P.D., *millivolts* .	1.0	1.5	2.0	2.0	3.0					
Calculated P.D., *millivolts*.	1.0	2.0	1.0	4.0	2.0					
Osmotic pressure, *mm.*...	6	6	7	9	11					

represent the differences between the E.M.F. readings obtained for the inside and outside solutions with the hydrogen electrode, corrected to 25°; in other words, the calculated P.D. values are calculated from the measurements of the hydrogen ion concentration according to the formula deduced by Donnan (which is identical with Nernst's formula).

The results of experiments on the effect of acidity on the P.D. are given in Table II.

The results show that with protein and acid alone the agreement between the observed and calculated values for the P.D. is fairly good except in the region near the isoelectric point, and the last experiment in Table II shows that agreement may be obtained in this range of pH also by the use of buffered solutions.

The effect of salt concentration on the P.D. was tested by a few experiments with NaCl, $CaCl_2$, and $LaCl_3$, and the results are given in Table III. Again the agreement between the observed and calcu-

TABLE III.

Effect of Different Chlorides on the P.D. *and Osmotic Pressure of 1 Per Cent Globulin Chloride.*

Concentration of salt.	0	N/1,024			N/256			N/32		
Salt		NaCl	$CaCl_2$	$LaCl_3$	NaCl	$CaCl_2$	$LaCl_3$	NaCl	$CaCl_2$	$LaCl_3$
pH inside	3.68	3.58	3.60	3.55	3.82	4.10	3.80	3.66	3.69	3.57
pH outside	3.34	3.36	3.38	3.33	3.76	3.93	3.73	3.63	3.63	3.54
Observed P.D., *millivolts*	21.0	13.5	12.5	13.0	4.0	3.0	3.5	1.0	1.0	1.0
Calculated P.D., *millivolts*	20.5	13.0	13.0	12.5	4.0	10.0	4.0	1.5	3.5	1.5
Osmotic pressure, *mm.*	52	25	26	28	20	16	19	4	4	4

TABLE IV.

Effect of pH on the P.D. *and Osmotic Pressure of 1 Per Cent Sodium Globulinate.*

pH inside	9.20	9.98	10.47	11.05
pH outside	9.81	10.34	10.85	11.31
Observed P.D., *millivolts*	−32.0	−23.0	−18.0	−11.5
Calculated P.D., *millivolts*	−36.5	−21.0	−22.5	−16.0
Osmotic pressure, *mm.*	220	174	165	152

lated values for the P.D. is sufficient to show that the P.D. is due to the Donnan equilibrium. Moreover, the different chlorides, at equivalent chloride ion concentrations, have identical effects in depressing the P.D. This proves that here too, as in the case of other proteins, the P.D. is affected only by the ion of opposite charge to that of the protein ion.

Table IV gives results which indicate that the predictions of the Donnan theory are fulfilled on the alkaline side of the isoelectric

[9] Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 617.

point. Here the pH inside was found to be less than the pH outside, and the observed P.D. was opposite in sign to that observed with the acid solutions, the inside solution now being negative with respect to the outside. While the quantitative agreement between the observed and calculated values is not so good as on the acid side, this is probably due to the effect of CO_2 from the air on the pH values. The solutions were protected by soda lime tubes while osmotic equilibrium was being reached, but were open to the air during the P.D. measurements.

IV.

Osmotic Pressure.

The osmotic pressure of these solutions was affected in the same sense by changes in pH as in the case of other proteins. On the acid side the osmotic pressure increased from small values near the isoelectric point to a maximum in the neighborhood of pH 3, and decreased with further increases in acidity. This is qualitatively in accord with the Donnan theory. The actual calculation of the osmotic pressure from the hydrogen ion measurements, however, gave values which were much higher than those observed, the maximum in the case of globulin chloride being over 3.5 times as high as that observed. The explanation for this discrepancy may lie in the existence of large aggregates in the globulin solutions. Loeb has shown[10] that the presence of undissolved particles of gelatin in place of dissolved gelatin has very little effect on the P.D. of a gelatin chloride solution, but that it materially decreases the osmotic pressure, the undissolved particles appearing to have but a slight share in the osmotic pressure as measured by the manometer. Since many of the globulin solutions were very opaque it is quite probable that much of the globulin was in the form of particles which produced no measurable osmotic pressure. However, a second preparation of globulin gave nearly clear solutions in HCl which still had an osmotic pressure much less than that calculated from the pH measurements.

The small osmotic pressure which was measured, however, was affected by salts in the way predicted by the theory. Table III shows that equivalent concentrations of different chlorides had the

[10] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 769.

same effect in decreasing this osmotic pressure, the decrease being evidently dependent on the concentration of the ion of opposite sign of charge to that of the protein ion.

The osmotic pressure observed in the experiments with alkali was more nearly of the same magnitude as that calculated on the basis of the Donnan theory, indicating that the sodium globulinate probably contained fewer large aggregates than the globulin chloride. Quantitatively, however, the agreement was poor; possibly this may be due to the presence of sodium carbonate.

SUMMARY.

1. The globulin prepared from ox serum by dilution and precipitation with carbon dioxide has been found, by electrometric titration experiments, to behave like an amphoteric electrolyte, reacting stoichiometrically with acids and bases.

2. The potential difference developed between a solution of globulin chloride, phosphate, or acetate and a solution of the corresponding acid, free from protein, separated from the globulin by a collodion membrane, was found to be influenced by hydrogen ion concentration and salt concentration in the way predicted by Donnan's theory of membrane equilibrium. In experiments with sodium globulinate and sodium hydroxide it was found that the potential difference could be similarly explained.

3. The osmotic pressure of such solutions could be qualitatively accounted for by the Donnan theory, but exhibited a discrepancy which is explicable by analogy with certain experiments of Loeb on gelatin.

4. The application of Loeb's theory of colloidal behavior, which had previously been found to hold in the case of gelatin, casein, egg albumin, and edestin, has thus been extended to another protein, serum globulin.

The writer's thanks are due to Dr. Jacques Loeb for his suggestion and guidance of this work.

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CATAPHORETIC CHARGES OF COLLODION PARTICLES AND ANOMALOUS OSMOSIS THROUGH COLLODION MEMBRANES FREE FROM PROTEIN.

BY JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 7, 1922.)

I.

When an aqueous salt solution is separated from pure water (both of originally the same hydrogen ion concentration) by a collodion membrane (not treated with a protein) a diffusion of water will occur from the side of the water to the side of the solution; and this diffusion is determined not only by the difference in the osmotic pressure on the opposite sides of the membrane, but in addition also by electrical forces, due to potential differences between membrane and liquid. In these experiments the salt solution is put into a collodion bag of about 50 cc. content, closed by a rubber stopper which is perforated by a glass tube serving as a manometer, as described in previous papers.[1] If the rise of level in the manometer observed after 20 minutes at 24°C. in mm. H_2O is plotted as ordinates over the salt concentration as abscissæ, we obtain the transport curves, which differ characteristically when the substance in solution is an electrolyte or a non-electrolyte. The transport curves for solutions of non-electrolytes, like cane-sugar, resemble those for electrolytes only in higher concentrations, M/8 or above, while for lower concentrations the transport curves for non-electrolytes and certain electrolytes are typically different, since the curves for these electrolytes show a rise followed by a drop (Figs. 2, 3, and 4) both of which are lacking in non-electrolytes. The drop is followed by a second rise coinciding with the rise observed in the cane-sugar curves, and the second rise may therefore be ascribed in both cases to the purely osmotic forces of the

[1] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 463, 621.

solution. The rise and fall of the curves at lower concentrations, however, which are only found in the case of the solutions of certain electrolytes (Figs. 2 and 3) and at definite hydrogen ion concentrations, must be ascribed to electrical forces.

In former papers the writer had arrived at the following formulation of these effects. When salt solutions are separated from pure water by collodion membranes (not treated with protein) the water is attracted by the solutions as if the water (in the pores of the membrane) were positively charged and as if it were attracted by the anion and repelled by the cation of the solution with a force which increases with the valency of the ion.

Girard,[2] and Bartell and Madison[3] had already suggested that two P.D. are active in this case, namely, first, a P.D. across the membrane between the salt solution and the water, and second, a P.D. inside the pore between the wall of the pore and the liquid diffusing through. These two P.D. are at right angles to each other. It seemed necessary to measure both P.D. at the same hydrogen ion concentrations of the solution in order to find out whether or not the transport curves can be explained on the basis of these P.D. If the P.D. across the membrane is designated as E and the P.D. inside the pore as ϵ, then, on the basis of Helmholtz's formula, the values of the electrical share of the transport curves should be parallel to the values of the product $E \times \epsilon$. It was shown in two preceding papers[1] that this was approximately true for gelatin membranes—or, more correctly, collodion membranes coated with gelatin on the solution side. These membranes behaved like gelatin membranes, the collodion acting only as a strengthening support of the gelatin film.

We intend to discuss in this paper the electrical part of the transport curves when the membranes are purely collodion membranes free from protein. The difference between the two cases is the following. When the membrane has a protein film, the charge of the water inside the pore (due to the formation of the electrical double layer) varies not only in quantity but in sign with the hydrogen ion

[2] Girard, P., *Compt. rend. Acad.*, 1908, cxlvi, 927; 1909, cxlviii, 1047, 1186; 1910, cl, 1446; 1911, cliii, 401; La pression osmotique et le méchanisme de l'osmose, Publications de la Société de Chimie-physique, Paris, 1912.

[3] Bartell, F. E., and Madison, O. E., *J. Physic. Chem.*, 1920, xxiv, 444, 593.

concentration, in a way as if the electrical double layer inside the pore were essentially determined by the Donnan equilibrium between gelatin and liquid. In the case of pure collodion membranes (free from protein), as used in the experiments for the present paper, the sign of charge of the water in the pores remains the same at any hydrogen ion concentration thus far used in the experiments. The water in the pores is practically always positively charged (except in the presence of salts with trivalent and probably tetravalent cations), while the collodion wall of the pores is negatively charged. This was ascertained by experiments on electrical endosmose[4] as well as by experiments on cataphoresis.[5]

When the water in the pores of a membrane assumes a positive charge, an electrical transport of water from the side of pure water into the solution can only take place when, in the P.D. across the membrane, the solution is negatively charged. This had already been suggested by Bartell.[3] We shall see that the results of the P.D. measurements agree with this suggestion and that they furthermore explain why the electrical transport of water into the solution increases with the valency of the anion of the salt used.

The value of ϵ, *i.e.* the P.D. between collodion and liquid, was determined by cataphoretic measurements taken from another paper in this number of this Journal.[5] The value of E, *i.e.* the P.D. across the membrane between solution and water, was measured at the beginning and the end of the diffusion experiments with the aid of a Compton electrometer and saturated KCl-calomel electrodes as described in the preceding papers. The transport curves for the same salt solutions vary with the hydrogen ion concentration. At the beginning of the experiment salt solutions and water were brought to the same pH, the pH being determined colorimetrically. When acid was required HCl was added, and when alkali was required KOH was used.

Fig. 1 gives the transport curves of water from the side of water into salt solutions at an initial pH of 3.0, the acid used being in all cases HCl. The ordinates of the curves are the rise of liquid in the manometers (connected with the solutions) after 20 minutes; the abscissæ

[4] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 563.
[5] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 109.

are the initial concentrations of the solutions. The curves in Fig. 1 are similar to the curves obtained with non-electrolytes like cane-sugar, and hence are entirely or chiefly due to the transport of water by purely osmotic forces. There is no indication of any transport due to electric forces.

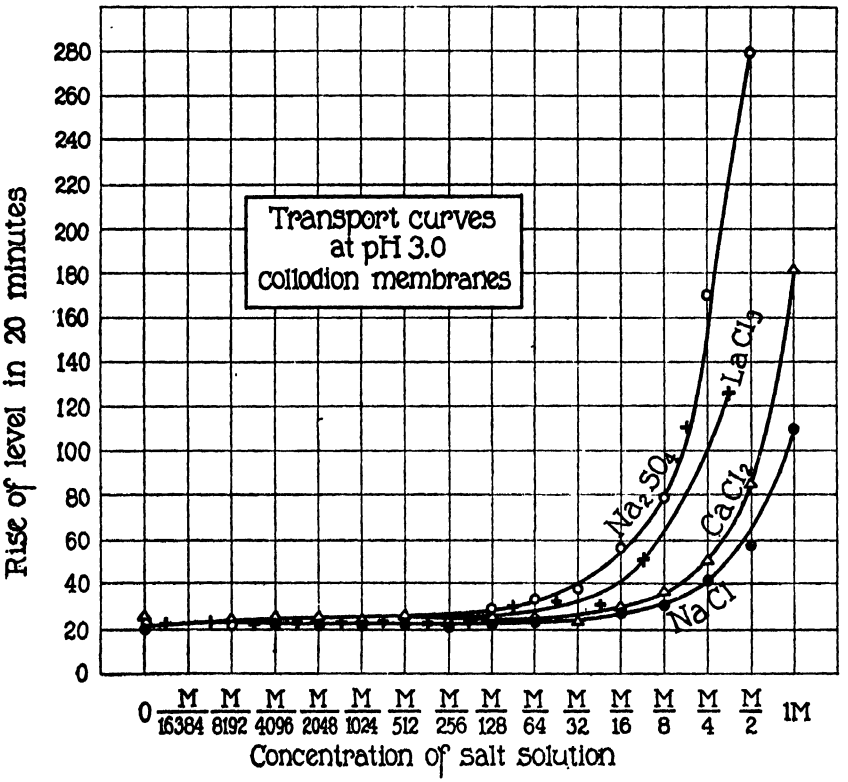

FIG. 1. Transport curves at pH 3.0 through collodion membranes. The ordinates are the rise of level in mm. H_2O in the manometers connected with the solution; the abscissæ are the concentrations of salt solutions. The curves show that at this pH the diffusion of water is determined purely by osmotic and not by electrical forces.

This result finds its explanation in the values given in Tables I, II, and III. In Tables I and II are given the P.D. across the membrane (E) at the beginning of the experiments and at the end (*i.e.*, after 20 minutes). The method of these measurements was described in a previous article. The solution was always positively charged with

TABLE I.

P.D. in millivolts between salt solutions and H_2O, of pH 3.0, across collodion membranes, *at beginning of experiment.* The minus or plus signs designate the sign of charge on the solution side of the membranes.

Concentration.....	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M	
Na_2SO_4.....	0	−1.0	+1.0	−1.0	−1.0	−1.5	− 2.5	− 3.5	− 5.0	− 6.5	− 9.0	−12.0	−16.0	−20.0		
KCl.....	+1.5		+0.5	0	+0.5	+0.5	+ 0.5	+ 0.5	+ 1.0	+ 1.0	+ 0.5	+ 1.5	+ 1.0	+ 1.5	+ 1.5	
NaCl.....	+1.0		+0.5	+1.5	+2.0	+3.0	+ 4.5	+ 7.0	+ 9.0	+11.0	+13.5	+16.0	+18.0	+21.0	+22.5	
LiCl.....	+1.0		+3.0	+3.0	+3.0	+5.0	+ 7.0	+10.0	+13.0	+17.5	+23.0	+27.0	+31.0	+34.0	+37.0	
$CaCl_2$.....	+2.0	+2.5	+4.0	+6.0	+7.0	+9.0	+13.0	+17.0	+22.0	+27.0	+31.0	+36.0	+39.0	+41.0	+48.0	
Concentration.....	0	M/49,152	M/24,576	M/12,288	M/6,144	M/3,072	M/1,536	M/768	M/384	M/192	M/96	M/48	M/24	M/12	M/6	M/3
$LaCl_3$.....	+2.0	+1.5	+2.0	+2.5	+3.0	+5.0	+ 6.0	+ 9.0	+13.5	+18.0	+23.0	+29.0	+34.0	+41.5	+48.0	+53.0

TABLE II.

P.D. in millivolts between salt solutions and H_2O, of pH 3.0, across collodion membranes, *at end of experiment.* The minus or plus signs designate the sign of charge on the solution side of the membranes.

Concentration.....	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M	
Na_2SO_4.....	+1.0	0	0	−1.5	−2.0	−3.5	− 5.0	− 6.0	− 6.0	− 6.5	− 7.0	−10.5	−11.0	− 9.0		
KCl.....	+0.5		+0.5	+0.5	0	0	+ 0.5	0	− 0.5	0	0	0	− 0.5	− 0.5	0	
NaCl.....	+1.0		+1.5	+1.0	+1.5	+2.0	+ 3.5	+ 6.5	+ 7.5	+ 9.0	+10.0	+11.0	+10.0		+ 7.0	
LiCl.....	+0.5		+1.0	+2.5	+3.0	+4.0	+ 6.5	+ 8.0	+12.5	+16.0	+19.0	+19.0	+18.0	+17.5	+14.5	
$CaCl_2$.....	+2.0	+3.0	+4.0	+5.0	+7.5	+9.0	+12.5	+16.5	+19.0	+22.0	+22.5	+22.5	+22.0	+21.0	+18.5	
Concentration.....	0	M/49,152	M/24,576	M/12,288	M/6,144	M/3,072	M/1,536	M/768	M/384	M/192	M/96	M/48	M/24	M/12	M/6	M/3
$LaCl_3$.....	+1.5	+2.0	+2.0	+2.0	+2.5	+3.0	+ 5.0	+ 9.0	+13.0	+18.0	+21.0	+23.5	+27.0	+25.0	+24.0	+25.0

the exception of the Na_2SO_4 solution; but at low concentrations of Na_2SO_4 the value of E was too small to have any effect, while at higher concentrations the value of ϵ became rather small. In Table III are given the cataphoretic P.D. (ϵ) between collodion particles and water in the same solutions as those mentioned in Tables I and II.

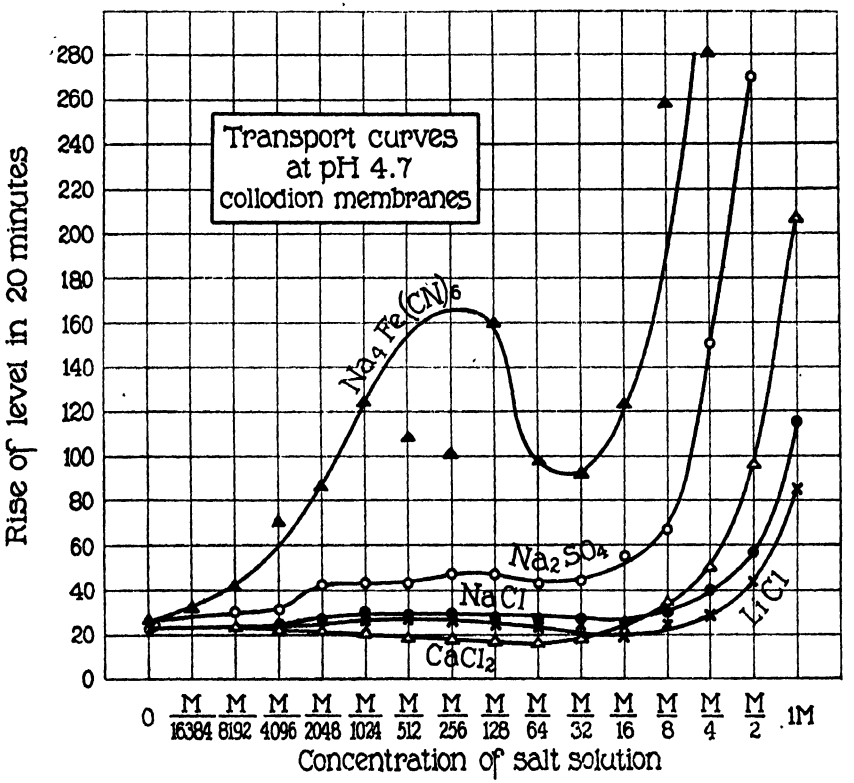

FIG. 2. Transport curves at pH 4.7. The transport curves for $Na_4Fe(CN)_6$ and Na_2SO_4 show a slight electrical effect in concentrations below M/32. The transport curves for NaCl, LiCl, and $CaCl_2$ show no electrical effect.

The method of the measurements of the cataphoretic P.D. was that described by Northrop.[6] As Table III shows, the sign of charge of the liquid in the pores of the membrane is also positive. Since the solution and the water inside the pores always have the same sign of charge (except in the case of Na_2SO_4 mentioned above), no

[6] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 629.

TABLE III.

Cataphoretic P.D. in millivolts between collodion particles and aqueous solutions at pH 3.0. The plus sign designates the charge of water.

Concentration.....	0	M/32,768	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4
Na_2SO_4.....	+63			+72	+72	+63	+73	+68	+65	+51	+46	+35	+28		+12
NaCl.....	+60			+62	+60	+57	+59	+59		+46	+39	+38	+29	+20	+14
$CaCl_2$.....	+62			+40	+37	+35	+34	+31	+27	+24	+19	+16	+14	+10	+ 7
$LaCl_3$.....	+68	+32	+28	+22	+18	+14	+ 8	+ 7	+ 5	+ 3	+ 1	0	0		

TABLE IV.

P.D. in millivolts between salt solutions and H_2O, of pH 4.7, across collodion membranes, *at beginning of experiment.* The minus or plus signs designate the sign of charge of the solutions.

Concentration.....	0	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M
$Na_4Fe(CN)_6$.....	+1.0	−8.0	−15.0	−21.0	−32.0	−29.0	−28.0	−27.5	−31.5	−32.5	−31.0	−40.0	−46.0	−52.5		
Na_2SO_4.....	+1.5		− 5.5	−10.0	−13.5	−15.0	−14.0	−14.0	−15.0	−17.5	−20.0	−22.0	−24.0	−27.5	−30.0	
KCl.....	−1.0			− 1.5	− 3.0	− 4.0	− 4.0	− 4.0	− 3.0	− 1.5	− 2.0	− 1.5	− 1.5	− 1.0	0	0
NaCl.....	+1.5			+ 4.5	+ 2.5	+ 6.5	+10.0	+11.0	+17.5	+21.0	+20.0	+27.0	+28.0	+31.0	+35.0	+35.0
LiCl.....	0			+ 4.0	+ 6.0	+ 9.5	+14.0	+19.0	+25.0	+33.0	+38.0	+45.0	+48.0	+54.0	+51.0	+59.0
$CaCl_2$.....	+2.5		+ 6.5	+ 9.0	+14.5	+21.0	+25.0	+31.5	+37.5	+43.5	+47.0	+52.0	+59.0	+61.5	+67.0	+67.0

TABLE V.

P.D. in millivolts between salt solutions and H_2O, of pH 4.7, across collodion membranes, *at end of experiment*. The minus or plus signs designate the sign of charge of the solutions.

Concentration.....	0	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M
$Na_4Fe(CN)_6$.....	−2.0	−12.5	−17.5	−25.0	−34.0	−34.0	−25.0	−24.0	−26.0	−21.5	−20.0	−18.5	−21.0	−25.0		
Na_2SO_4.....	−4.0		− 7.5	−14.0	−15.0	−13.5	−17.0	−12.5	−12.5	−11.5	−11.0	−11.5	−11.0	−11.5	−12.5	
KCl.....	−8.0			− 7.0	− 8.0	− 7.5	− 8.0	− 4.0	− 3.0	− 3.0	− 1.5	− 0.5	0	0	0	0
NaCl.....	−1.0			− 1.5	− 2.0	+ 1.5	+ 5.0	+ 4.5	+ 9.0	+10.0	+ 9.5	+10.0	+ 9.0	+10.0	+ 8.5	+ 7.5
LiCl.....	−4.0			− 2.5	− 2.5	− 1.5	+ 7.5	+14.0	+18.5	+19.0	+21.0	+20.5	+20.0	+19.0	+17.0	+17.0
$CaCl_2$.....	−1.0		+ 2.5	+ 4.0	+10.0	+ 9.0	+17.5	+22.0	+24.5	+24.0	+23.5	+24.0	+23.5	+20.5	+20.5	+20.5

TABLE VI.

Cataphoretic P.D. in millivolts between collodion particles and aqueous solutions at pH 4.7. The plus sign designates the charge of water.

Concentration.....	0	M/32,768	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2
$Na_4Fe(CN)_6$.....	+29	+51	+55	+60	+64	+68	+65	+57	+50	+42	+30	+21	+13	+ 9		
Na_2SO_4.....	+28	+47	+47	+56	+59	+65	+70	+69	+63	+56	+43	+36	+26	+19	+13	
KCl.....	+32	+31	+37	+45	+52	+53	+62	+66	+66	+60	+49	+40	+30	+21	+14	
NaCl.....	+26			+47	+53	+58	+60	+64	+61	+55	+49	+37	+30	+20	+14	+10
LiCl.....	+31	+41	+42	+48	+47	+57	+60	+64	+68	+61	+55	+44	+36	+22	+17	
$CaCl_2$.....	+36		+30	+31	+32	+35	+32	+28	+26	+22	+17	+14	+10	+ 8	+ 6	
$LaCl_3$.....	+29	+17	+19	+19	+21	+14	+10	+ 8	+ 4	+ 4	0	0	− 3	− 6		

electrical transport of water into the solution should be possible, as was found to be the case.

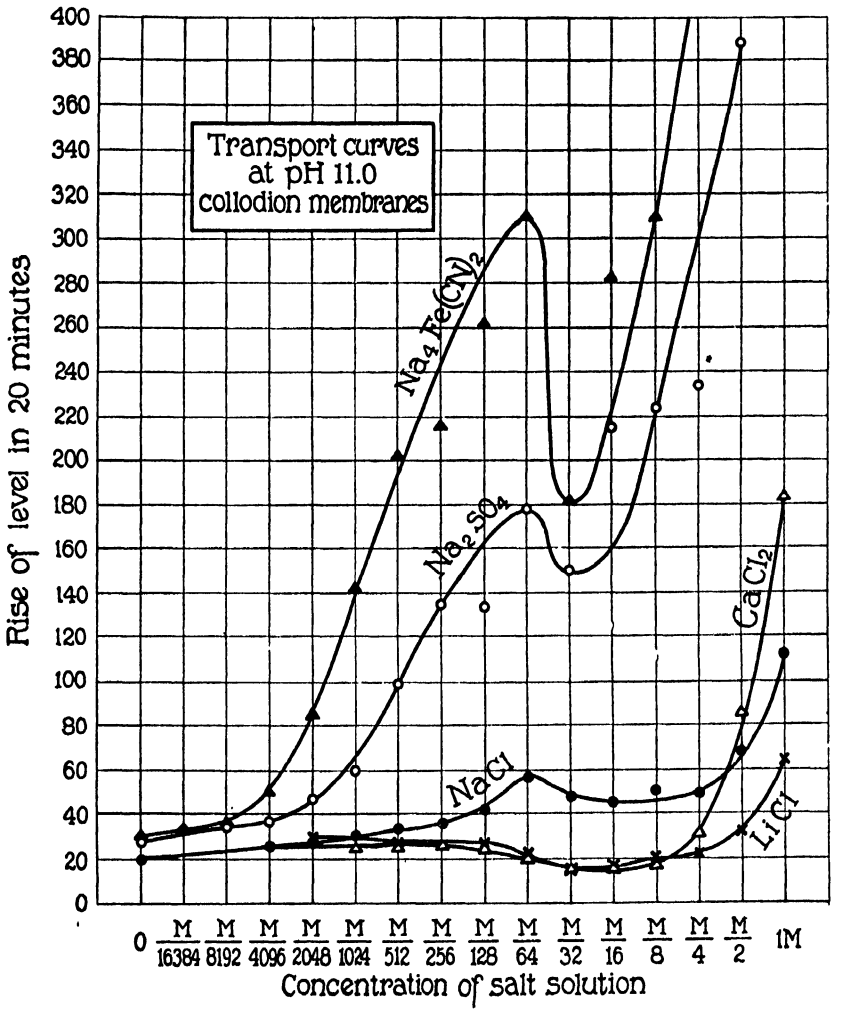

FIG. 3. Transport curves at pH 11.0. The electrical effect is visible only for NaCl, Na_2SO_4, and $Na_4Fe(CN)_6$ for concentrations below M/64.

Fig. 2 gives the transport curves at a lower hydrogen ion concentration; namely, pH 4.7. We now notice a marked electrical effect in the transport curves for $Na_4Fe(CN)_6$ and a slight one for Na_2SO_4. This is accounted for in Tables IV, V and VI. Tables IV and V show that

TABLE VII.

P.D. in millivolts between salt solutions and H_2O, of pH 11.0, across collodion membranes, *at beginning of experiment.* The minus or plus signs designate the sign of charge on the solution side of the membranes.

Concentration.....	0	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M
$Na_4Fe(CN)_6$.....	−1.5	−2.5	−5.0	−8.5	−13.0	−19.0	−22.5	−30.0	−27.0	−30.5	−29.0	−37.5	−43.0	−47.5		
Na_2SO_4.....	−2.0		−4.5	−6.0	− 8.0	−14.0	−17.0	−18.0	−17.0	−18.0	−17.0	−19.0	−21.0	−24.0	−29.0	
KCl.....	0			−2.5	− 3.0	− 3.0	− 5.0	− 4.0	− 4.0	− 3.5	− 2.5	− 2.5	− 1.5	− 1.5	− 1.0	− 1.0
NaCl.....	0			−1.5	− 2.0	− 2.5	− 1.5	− 1.5	+ 1.0	+ 2.0	+ 6.0	+10.0	+13.0	+16.0	+19.0	+22.0
LiCl.....	−0.5			−1.5	− 2.5	− 1.5	0	+ 3.5	+ 7.5	+13.0	+18.0	+23.0	+27.0	+32.5	+38.0	+41.0
$CaCl_2$.....	−2.5		−2.5	−2.0	− 1.0	+ 1.0	+ 5.0	+10.0	+15.0	+20.0	+26.0	+31.5	+37.0	+42.0	+46.5	+51.0

TABLE VIII.

P.D. in millivolts between salt solutions and H_2O, of pH 11.0, across collodion membranes, *at end of experiment.* The minus or plus signs designate the sign of charge on the solution side of the membranes.

Concentration.....	0	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M
$Na_4Fe(CN)_6$.....	−3.0	−3.5	−5.0	−7.5	−12.0	−20.0	−25.0	−24.0	−26.0	−25.0	−21.0	−22.5	−23.0	−24.0		
Na_2SO_4.....	−2.5		−4.5	−6.5	− 6.5	−13.5	−16.0	−18.0	−16.0	−16.0	−14.0	−15.0	−14.0	−13.0	−19.0	
KCl.....	−2.0			−2.0	− 2.5	− 3.0	− 4.0	− 4.0	− 3.5	− 3.0	− 2.5	− 2.0	− 1.0	− 0.5	0	0
NaCl.....	−0.5			− 2.0	− 2.0	− 3.0	− 2.5	− 1.5	− 0.5	+ 1.5	+ 1.5	+ 6.0	+ 7.0	+ 7.5	+ 7.5	+ 8.0
LiCl.....	−2.5			− 3.5	− 2.5	− 2.5	− 1.0	+ 3.0	+ 7.5	+10.0	+13.0	+17.0	+18.0	+16.5	+13.0	+15.0
$CaCl_2$.....	−2.0		−2.5	−3.0	− 1.5	− 1.0	+ 5.0	+ 8.5	+13.5	+16.5	+19.5	+22.0	+21.0	+23.0	+20.0	+18.0

TABLE IX.

Cataphoretic P.D. in millivolts between collodion particles and aqueous solutions at pH 11.0. The plus sign designates the sign of charge of the water.

Concentration	0	M/32,768	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4
$Na_4Fe(CN)_6$	+59	+64	+66	+74	+71	+71	+70	+69	+52	+41	+32	+24	+16		
Na_2SO_4	+60	+69	+70	+70	+71	+73	+75	+65	+63	+56	+49	+35	+28	+20	
NaCl	+62		+62	+66	+65	+66	+65	+66	+65	+62	+53	+43	+34	+24	+18
$CaCl_2$	+58	+48	+48	+40	+39	+37	+34	+29	+28	+24	+19	+15	+ 9	+ 6	

the solutions of $Na_4Fe(CN)_6$ and Na_2SO_4 assume a considerable negative charge in the P.D. across the membrane and that this P.D. is greater in the case of $Na_4Fe(CN)_6$ than in the case of Na_2SO_4. Since, according to Table VI, the water in the pores is positively charged, an electrical transport must occur. In KCl there is a slight negative charge of the solution (Table IV) but too small to have any effect on the transport of water. In the case of NaCl, LiCl, $CaCl_2$, and $LaCl_3$ no electrical transport is possible since the solution as well as the water in the pores are positively charged.

Observations taken at pH 5.8 were similar to those at pH 4.7 except that the effects of Na_2SO_4 and $Na_4Fe(CN)_6$ were greater than at pH 4.7. The P.D. across the membrane was also greater for these salts. Since otherwise everything was similar to the result at pH 4.7, further discussion of the results at pH 5.8 may be omitted.

Fig. 3 gives the transport curves at pH 11.0, the solutions being brought to this pH by the addition of KOH. $Na_4Fe(CN)_6$, Na_2SO_4, NaCl, and KCl (not shown in the figure) gave electrical transport curves. In this case the salt solutions mentioned were negatively charged (Tables VII and VIII), while the water in the pores was as usual positively charged (Table IX); the negative charge in the solution (Tables VII and VIII) increased in the order of KCl and NaCl $<$ Na_2SO_4 $<$ $Na_4Fe(CN)_6$, as did the electrical part of the transport curves in Fig. 3. In the case of LiCl and $CaCl_2$ the solutions were positively charged and hence the transport curves are the effect of the osmotic forces alone.

The P.D. measurements therefore explain the transport curves in a semiquantitative way in all these experiments.

II.

While all these results show that the electrical transport of water in these experiments varies semiquantitatively with the value of the product $E \times \epsilon$, some difficulties are encountered when we compare the effect of Na_2SO_4 or $Na_4Fe(CN)_6$ on the electrical transport at pH 11.0 with that at pH 5.8. In Fig. 4 are presented the transport curves for Na_2SO_4 at different pH, varying from 2.0 to pH 12.0.

At pH 2.0 and pH 3.0 the P.D. across the membrane (Table X) is small, and this accounts for the fact that at both pH the transport

curves are purely osmotic in character (Fig. 4). At pH 4.7 the electrical transport of liquid from water into the Na_2SO_4 solution

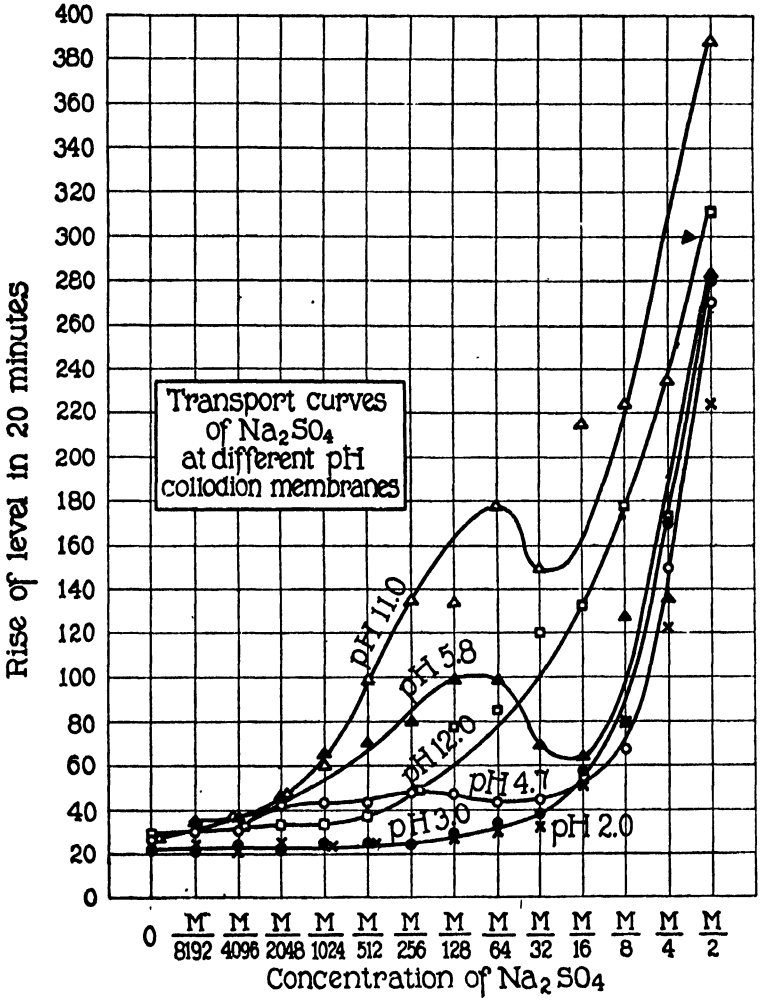

FIG. 4. Transport curves for Na_2SO_4 at different pH showing that the electrical effect for the same salt varies considerably with the hydrogen ion concentration of the solution.

becomes noticeable, as already mentioned. At pH. 5.8 (Fig. 4) the electrical transport of water from the side of pure water into the Na_2SO_4 solution is greater than at pH 4.7, and at pH 11.0

TABLE X.

P.D. in millivolts between solutions of Na_2SO_4 and H_2O of same pH across a collodion membrane, *at beginning of experiment.* The plus or minus sign designates the sign of charge of the solutions.

Concentration of Na_2SO_4	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2
pH 2.0	+0.5	+ 0.5	+ 0.5	+ 0.5	+ 1.0	+ 1.0	+1 .0	+ 1.0	+ 1.0	0	− 1.0	− 4.0	− 7.0	−12.0
pH 3.0	0	− 1.0	+ 1.0	− 1.0	− 1.0	− 1.5	− 2.5	− 3.5	− 5.0	− 6.5	− 9.0	−12.0	−16.0	−20.0
pH 4.7	+1.5	− 5.5	−10.0	−13.5	−15.0	−14.0	−14.0	−15.0	−17.5	−20.0	−22.0	−24.0	−27.5	−30.0
pH 5.8		−20.0	−21.0	−30.0	−29.0	−23.0	−22.0	−24.0	−21.0	−15.0	−24.0	−30.0	−26.0	−34.0
pH 11.0	−2.0	− 4.5	− 6.0	− 8.0	−14.0	−17.0	−18.0	−17.0	−18.0	−17.0	−19.0	−21.0	−24.0	−29.0
pH 12.0	0	− 0.5	− 1.0	− 1.0	− 1.0	− 1.5	− 3.0	− 4.0	− 5.5	− 8.0	−10.5	−13.5	−16.5	−21.0

TABLE XI.

Cataphoretic P.D. in millivolts between collodion particles and Na_2SO_4 solutions at different pH. The plus sign designates the sign of charge of the water.

Concentration of Na_2SO_4	0	M/32,768	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4
pH 2.0	+37	+43	+43	+54	+41	+40	+42	+41	+42	+38	+32	+25	+19	+10	
pH 3.0	+63			+72	+72	+63	+73	+68	+65	+51	+46	+35	+28	(+6)	+12
pH 4.7	+28	+47	+47	+56	+59	+65	+70	+69	+63	+56	+43	+36	+26	+19	+13
pH 5.8	+21	+50	+58	+53	+60	+64	(+90)	+67	+62	+55	+46	+36	+27	+21	
pH 11.0	+60	+69	+70	+70	+71	+73	+75	+65	+63	+56	+49	+35	+28	+20	
pH 12.0	+45	+47	+50	+50	+49	+52	+50	+49	+47	+42	+37	+31	+25	+19	

(Fig. 4) the electrical transport of water is still greater than at pH 5.8. At pH 12.0 (Fig. 4) the electrical transport diminishes again. The fact that the electrical transport is less at pH 12.0 than at pH 11.0 finds its explanation in Table X showing that the P.D. across the membrane is considerably smaller at pH 12.0 than at pH 11.0.

A difficulty arises, however, if we wish to account for the fact that the electrical transport curve for Na_2SO_4 is higher at pH 11.0 than that at pH 5.8, since the P.D. across the membrane is greater at pH 5.8 than at pH 11.0 (Table X), while Table XI shows that the value of ϵ is practically identical at both pH.

The same difficulty occurs in the case of the transport curves for $Na_4Fe(CN)_6$ at pH 5.8 and 11.0, these transport curves being higher at pH 11.0 than at pH 5.8, although the values $E \times \epsilon$ do not warrant the difference in the transport curves. It seems to follow that some other variable in the equation for the diffusion of water (besides E and ϵ) is affected by the pH. The low value of the electrical transport in Na_2SO_4 solutions at pH 2.0 is accounted for by the low value of the P.D. across the membrane (Table X).

The electrical part of the transport curves lies in a concentration of the salts between zero and about M/8 or M/4. The transport curves for $Na_4Fe(CN)_6$ or Na_2SO_4 rise at first with increasing concentration until a maximum is reached at a molecular concentration of between M/32 or M/16, then drop slightly, and then rise again sharply (Figs. 2, 3, and 4). This latter rise is probably due exclusively to the osmotic effect of the salt in solution and need not be considered for our present purpose. The initial rise in the curves with increasing concentration of Na_2SO_4 or $Na_4Fe(CN)_6$ is, however, electrical in character and is accounted for by a corresponding rise in the value of E with increasing concentration, as is shown in Tables IV, VII, and X.

The drop of the electrical transport curves at a concentration of between M/32 and M/16 at pH 5.8 and pH 11.0 is explained by a corresponding drop in values of ϵ as is shown in Tables VI and IX. We can therefore say that from the influence of electrolytes on the P.D. across the membrane (E) and on the cataphoretic P.D. (ϵ) it is possible to explain semiquantitatively the phenomena of anomalous osmosis through a collodion membrane with the exception of some minor discrepancies.

III.

We may finally inquire into the nature of the P.D. across the membrane. It was pointed out that it was essentially, but perhaps not exclusively, a diffusion potential due to the difference in the rate of migration of the oppositely charged ions of a salt through the membrane.

The liquid in the pores of a collodion membrane is generally positively charged; only solutions of salts with trivalent (and probably tetravalent) cations can, in sufficiently high concentrations, cause the liquid to be negatively charged. If we omit these exceptions, it follows that an electrical transport of liquid through the membrane from the side of the water into the solution can only occur when in the P.D. across the membrane a sufficiently large negative charge is found on the solution side. The experiments show that this occurs in a pronounced way only when the anion of the salt is plurivalent, *i.e.* SO_4, oxalate, PO_4, $Fe(CN)_6$, etc., while the cation is univalent, and the question arises, why this should be so. The answer is given by the measurements of diffusion potentials of solutions of salts against pure water of the same pH without the interposition of a membrane, which show that in the diffusion potentials of solutions of salts with plurivalent anions and univalent cations against water, the solutions assume a negative charge which increases with the valency of the anions and the concentration of the salts. This is obvious from the results in Table XII giving the diffusion potentials for a series of salt solutions against water without the interposition of a membrane at pH 4.7. When the anion is plurivalent and the cation univalent, the solution assumes a negative charge in the P.D. across the membrane and the negative charge increases with the valency of the anion and the concentration of the salt. The same is true for the charge of the solution when the diffusion occurs through a collodion membrane. Since the salts can diffuse through the collodion membrane, diffusion potentials are bound to occur and we cannot doubt that these diffusion potentials explain the peculiar effect which the sign of charge and valency of ion have on the electrical part of the transport curves; *i.e.*, on anomalous osmosis. The P.D. across the membrane is essentially due to the diffusion potential of the solution.

TABLE XII.

Diffusion potentials in millivolts between salt solutions and H_2O of pH 4.7.

Concentration	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M
$Na_4Fe(CN)_6$	−2.5	− 4.0	− 7.0	−10.0	−14.0	−18.0	−24.0	−27.0	−32.0	−36.5	−43.0	−49.0	−55.0		
Na_2SO_4		− 2.5	− 4.0	− 5.5	− 7.0	− 9.0	−11.5	−13.5	−15.5	−18.0	−23.0	−26.0	−29.0	−34.0	
KCl		0	+ 1.0	+ 0.5	+ 0.5	+ 0.5	+ 0.5	0	0	0	0	0	0	0	0
NaCl	0	+ 7.0	+ 9.0	+11.5	+14.0	+18.0	+20.5	+22.5	+25.0	+27.0	+29.5	+33.5	+36.0	+39.0	+42.0
LiCl	0	+ 6.0	+ 9.5	+16.5	+20.5	+23.5	+30.0	+37.5	+42.5	+46.5	+50.0	+55.0	+60.0	+63.5	+68.5
$CaCl_2$		+16.0	+21.0	+26.5	+31.0	+35.0	+41.0	+47.0	+53.0	+59.0	+64.5	+70.0	+76.0	+78.0	+84.0
$BaCl_2$	+ 6.5	+10.0	+15.0	+20.0	+26.0	+31.5	+36.5	+43.0	+47.5	+52.5	+57.0	+61.5	+67.5	+75.0	
$MgCl_2$	+ 7.0	+11.0	+17.5	+24.0	+30.0	+36.0	+41.0	+46.5	+53.5	+62.0	+68.0	+72.5	+78.5	+86.5	+96.5
$MgSO_4$	+ 5.0	+ 6.0	+ 6.0	+ 7.5	+ 7.5	+ 8.5	+ 9.0	+10.0	+11.0	+11.0	+11.0	+11.5	+11.0	+10.0	
$LaCl_3$	+10.0	+14.0	+19.0	+24.0	+29.0	+36.0	+43.0	+49.0	+55.0	+61.0	+67.0	+74.0	+80.0	+88.0	

TABLE XIII.

P.D. in millivolts across the membrane minus diffusion potential without membrane, at pH 4.7.

Concentration	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4
$Na_4Fe(CN)_6$	−11.0	−14.0	−22.0	−15.0	−10.0	−3.5	−4.5	− 0.5	+5.5	+ 3.0	+ 3.0	+ 2.5
Na_2SO_4	− 3.0	− 6.0	− 8.0	− 8.0	− 5.0	−2.5	−1.5	− 2.0	−2.0	+ 1.0	+ 2.0	+ 1.5
NaCl		− 4.5	− 9.0	− 7.5	− 8.0	−9.5	−5.0	− 4.0	−7.0	− 2.5	− 5.5	− 5.0
$CaCl_2$	− 9.5	−12.0	−12.0	−10.0	−10.0	−9.5	−9.5	− 9.5	−12.0	−12.5	−11.0	−14.5

While the diffusion potentials of salt solutions against water without a membrane agree to a large extent with the P.D. across the collodion membrane, the two P.D. are by no means identical. If the diffusion potentials without interposition of a membrane in Table XII are compared with the P.D. across the collodion membrane at pH 4.7 given in Table IV, it is noticeable that the values for diffusion potentials differ in a definite sense from the values for the P.D. across the membrane, especially for the concentrations below M/128 or M/256. This difference between P.D. across the membrane minus diffusion potential without a membrane has in this case always a negative value, as is shown in Table XIII. It looks as if the rate of diffusion of the anions was comparatively more retarded by the membrane than the rate of diffusion of the cations. Since the collodion membrane is negatively charged, it might be argued that the retardation is due to a repulsion of the anions by the negatively charged membrane and an attraction of the positive ion of the salt. While this is possible, it is also possible that another P.D. is superposed upon the diffusion potential. It will be necessary to return to this subject in a subsequent paper.

SUMMARY AND CONCLUSIONS.

1. It had been shown in previous papers that when a salt solution is separated from pure water by a collodion membrane, water diffuses through the membrane as if it were positively charged and as if it were attracted by the anion of the salt in solution and repelled by the cation with a force increasing with the valency. In this paper, measurements of the P.D. across the membrane (E) are given, showing that when an electrical effect is added to the purely osmotic effect of the salt solution in the transport of water from the side of pure water to the solution, the latter possesses a considerable negative charge which increases with increasing valency of the anion of the salt and diminishes with increasing valency of the cation. It is also shown that a similar valency effect exists in the diffusion potentials between salt solutions and pure water without the interposition of a membrane.

2. This makes it probable that the driving force for the electrical transport of water from the side of pure water into solution is primarily a diffusion potential.

3. It is shown that the hydrogen ion concentration of the solution affects the transport curves and the diffusion potentials in a similar way.

4. It is shown, however, that the diffusion potential without interposition of the membrane differs in a definite sense from the P.D. across the membrane and that therefore the P.D. across the membrane (E) is a modified diffusion potential.

5. Measurements of the P.D. between collodion particles and aqueous solutions (ϵ) were made by the method of cataphoresis, which prove that water in contact with collodion particles free from protein practically always assumes a positive charge (except in the presence of salts with trivalent and probably tetravalent cations of a sufficiently high concentration).

6. It is shown that an electrical transport of water from the side of water into the solution is always superposed upon the osmotic transport when the sign of charge of the solution in the potential across the membrane (E) is opposite to that of the water in the P.D. between collodion particle and water (ϵ); supporting the theoretical deductions made by Bartell.

7. It is shown that the product of the P.D. across the membrane (E) into the cataphoretic P.D. between collodion particles and aqueous solution (ϵ) accounts in general semiquantitatively for that part of the transport of water into the solution which is due to the electrical forces responsible for anomalous osmosis.

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THE INFLUENCE OF ELECTROLYTES ON THE CATAPHORETIC CHARGE OF COLLOIDAL PARTICLES AND THE STABILITY OF THEIR SUSPENSIONS.

I. EXPERIMENTS WITH COLLODION PARTICLES.

BY JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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I.

INTRODUCTION.

The work of McTaggart[1] on the electrification of water-gas surfaces is of fundamental importance for the question concerning the origin of the potential differences responsible for the cataphoresis of solid particles in aqueous solutions. McTaggart found that the P.D. between gas bubbles and water is about 55 millivolts, the water assuming a positive and the bubble a negative charge. The nature of the gas had apparently no influence on the P.D. and even particles of ice moving in water had the same P.D. From this he concludes that these cataphoretic P.D. are determined exclusively by the water. When the bubble moves, one surface of the double layer is fixed to the gas bubble and "preserves its identity more or less as it moves through the liquid." This layer must have an excess of OH ions when the bubble assumes its usual negative charge. He also points out that "the fact that so many substances in contact with water are negatively charged indicates that water by itself produces an electrification upon which is superposed an electrification due to the substance in contact with it."

On the basis of experiments on waterfall electricity Lenard[2] also arrived at the conclusion that the cause for the formation of an

[1] McTaggart, H. A., *Phil. Mag.*, 1914, xxvii, 297; xxviii, 367.

[2] Lenard, P., *Ann. Physik*, 1915, xlvii, 463.

electrical double layer at water surfaces must be in the water itself, and that both strata of the double layer between a colloidal particle and water must lie entirely in the water. Waterfall electricity is due to the mechanical tearing off of very minute particles of water from the surface. Lenard observed that when the particles torn off were very minute they were negatively charged, while when they were larger they were electrically neutral. According to McTaggart it is, however, not entirely certain that the double layer which surrounds a bubble of air in water and is active in a cataphoresis experiment is the same as that which is responsible for waterfall electricity.

The question arises: What causes this ionic stratification near the surface of the liquid? Since salts raise the surface tension of water, they must be contained in the surface in lower concentration than in the body of liquid. This was already emphasized by McTaggart. Should it be possible that the OH and H ions have a different effect on the surface tension, the H ions causing a greater rise of the surface tension of water than the OH ions? In that case the concentration of the OH ions should be higher in the outermost, *i.e.*, most superficial, stratum of the surface of the water than the concentration of the H ions, while the concentration of H ions should be greater in the layer of water underneath. This would explain why water generally assumes a positive charge in contact with colloidal particles, provided that the most superficial layer of water adheres to the particle moving in the electric field. It is, however, not necessary to make any definite assumption in regard to the nature of the forces responsible for the ionic stratification beyond stating that they must be "molecular forces" *inherent in the water itself*.

There are, however, cases where the cataphoretic P.D. seems to have a different origin from that of air bubbles moving in an electric field. This is apparently true for protein particles where the charge is obviously connected with the ionization of the particles. Thus it is well known through the experiments of Hardy, Michaelis,[3] and others that such particles do not migrate in an electric field at the hydrogen ion concentration of their isoelectric point, while they migrate towards the cathode when the hydrogen ion concentration is higher and

[3] Michaelis, L., Die Wasserstoffionenkonzentration, 2nd edition, Berlin, 1922.

towards the anode when the hydrogen ion concentration is lower than that of the isoelectric point of the protein. It is obvious that the ionization of these particles controls their cataphoretic P.D.

It seemed of interest to compare the influence of neutral salts on these two types of cataphoretic potentials. A convenient material was obtained in the form of fine collodion particles which were found to behave cataphoretically very much like air bubbles. When such collodion particles were coated with a protein, *e.g.*, gelatin, they could be used for the study of the cataphoretic potentials of the protein with which the particles were coated. In this paper we shall report only on the experiments with pure collodion particles *free from contamination with protein.*

The method of the evaluation of the cataphoretic P.D. between small particles and aqueous solutions was based on mobility measurements of individual particles by cataphoresis under the microscope, in which the apparatus with non-polarizable electrodes described by Northrop[4] was used. The apparent depth of the cell for the mobility measurements was 0.6 mm. and the time required for a particle to travel a distance of 45μ or when necessary 90μ was measured for four depths; namely, 0, 0.1, 0.2, and 0.3 mm., to eliminate the influence of the cataphoresis of the water itself. These measurements were averaged and the mobility in centimeters per second per volt per centimeter $\times$ 10^{-4} was calculated. By multiplying this mobility by the factor 14, the P.D. between the particles and the medium was obtained in millivolts (for a temperature of 24°C.). The details for this calculation may be gathered from Burton[5] and Ellis.[6] The strength of the electric field in our experiments was 4.5 volts per centimeter.

II.

Experiments on the Cataphoretic P.D. *of Collodion Particles.*

The collodion particles were prepared in the following way. Merck's solution of non-flexible collodion in alcohol and ether was

[4] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 629.

[5] Burton, E. F., The physical properties of colloidal solutions, 2nd edition, London, New York, Bombay, Calcutta, and Madras, 1921.

[6] Ellis, R., *Z. physik. Chem.*, 1911–12, lxxviii, 321; 1912, lxxx, 597.

poured into H_2O and stirred with a glass rod. A spongy mass of solid collodion accumulated around the rod. The solid collodion was washed a few times with H_2O and dried with filter paper. A strong solution of this collodion was then made up in chemically pure acetone (about 10 gm. in 100 cc.) and enough H_2O was added until a light cloudiness was formed. The acetone was then removed from the solution by distillation under reduced pressure, and the remaining milky fluid was centrifuged. The sediment, when stirred up with H_2O, gives a concentrated suspension of collodion particles. The suspension was centrifuged and the larger particles in the sediment were used. These particles were very convenient for the investigation of the migration as well as of the stability of the suspension.

In the following experiments 2 drops of the concentrated suspension of collodion particles were added to 50 cc. of the various solutions used, and this dilute suspension served as material for the mobility measurements. The collodion particles moved towards the anode, thus indicating that they were negatively charged. Only in the presence of salts with trivalent cations, like $LaCl_3$, was the sign of charge reversed.

In these experiments it was found that the P.D. between the collodion particles and water was a minimum when the water contained no salts and was as near as possible the point of neutrality. When acid, alkali, or a salt was added the P.D. rose rapidly with increasing concentration until a maximum was reached. This maximum depended on the nature of the electrolyte added. It was high when the anion was plurivalent and the cation univalent, and low when the cation was plurivalent and the anion monovalent. The influence of acid, alkali, and salts with monovalent ions; *e.g.*, HCl, NaOH, and NaCl, was not very different. Upon the addition of increasing concentrations of electrolytes the P.D. rose, reaching a maximum, and then dropped again upon a further rise of concentration. We will illustrate this in more detail.

The distilled water used in these experiments had a pH of about 5.8. At this pH the cataphoretic P.D. between collodion particles and water was about 22 to 30 millivolts. It was difficult to obtain more definite figures probably on account of the CO_2 error. When the distilled water was replaced by acid or alkali of different concen-

trations, the P.D. rose at first with increasing concentrations of the acid or alkali until a maximum was reached at a concentration between M/1,000 and M/500. This is shown in Fig. 1, where the ordinates

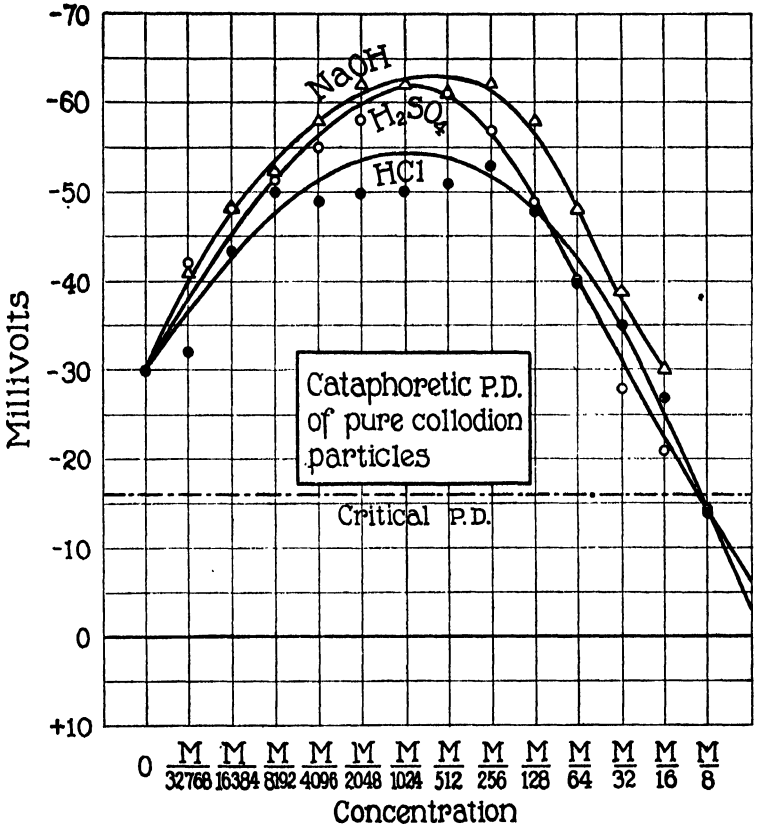

FIG. 1. Influence of acid and alkali on the cataphoretic P.D. between collodion particles and water. The original pH of water was about 5.0. The abscissæ are the concentrations of acids or alkali, the ordinates are the P.D. in millivolts. The collodion particles were negatively charged. The line marked "Critical P.D." (at 16 millivolts) is in this and the following figures the P.D. below which the collodion suspension is no longer stable.

are the P.D. and where the abscissæ are the molar concentrations of the electrolytes. The P.D. was at the maximum about 55 to 65 millivolts. With a further rise in concentrations of acid or alkali the P.D. dropped rapidly and the drop was about the same for the

acid and alkali (Fig. 1). The rise in P.D. must be due to the fact that in the case of low concentrations of NaOH an excess of OH ions and in the case of low concentrations of HCl and H_2SO_4 an excess of Cl and SO_4 ions respectively is forced into the outermost layer of water at the surface collodion-water, and that this layer moves with the collodion particles in an electric field.

It seems worthy of notice that the maximal P.D. was about the same in the case of H_2SO_4 and of NaOH, namely about 63 millivolts at a molecular concentration of electrolyte of about 1/1,024, while it was a little lower in the case of HCl; namely, about 55 millivolts. It may be worth while to point out that the P.D. between air bubbles and water in McTaggart's experiments was about 55 millivolts; though he does not state at which hydrogen ion concentration this P.D. was observed. The order of magnitude of the cataphoretic P.D. of air bubbles and of collodion particles in water is, therefore, not materially different so that we may say that the cataphoretic P.D. between collodion particles and water has in all probability the same origin as in McTaggart's experiments; namely, in molecular forces inherent in the water itself which push the cations of an electrolyte deeper down into the surface layer, than the anions. The collodion particles increase this effect only slightly, since the maximal P.D. between collodion particles and water is only about 70 millivolts. Such a P.D. was observed in the experiments recorded in Fig. 2.

Fig. 2 gives the results of measurements of the P.D. between the collodion particles and solutions of five different salts, $Na_4Fe(CN)_6$, Na_2SO_4, NaCl, $CaCl_2$, and $LaCl_3$, all solutions having a pH of 5.8. This experiment was intended to illustrate the relative influence of the valency of the anions and cations on the cataphoretic P.D. The P.D. rose upon the addition of salts with univalent cation (Na) to a maximal value of about 70 millivolts, but the concentration required to reach this maximum was least for $Na_4Fe(CN)_6$, a little higher for Na_2SO_4, and still a little higher for NaCl. A further increase of the concentration of the salts depressed the P.D., the curves dropping rapidly (Fig. 2). The maximum was but slightly higher in the case of $Na_4Fe(CN)_6$ than in the case of Na_2SO_4, and but slightly higher in the case of Na_2SO_4 than in the case of NaCl.

Fig. 2 shows also that the initial rise in the P.D. did not occur at all or occurred at a very low concentration when the salt added was $LaCl_3$, and that the rise was small when the salt added was $CaCl_2$;

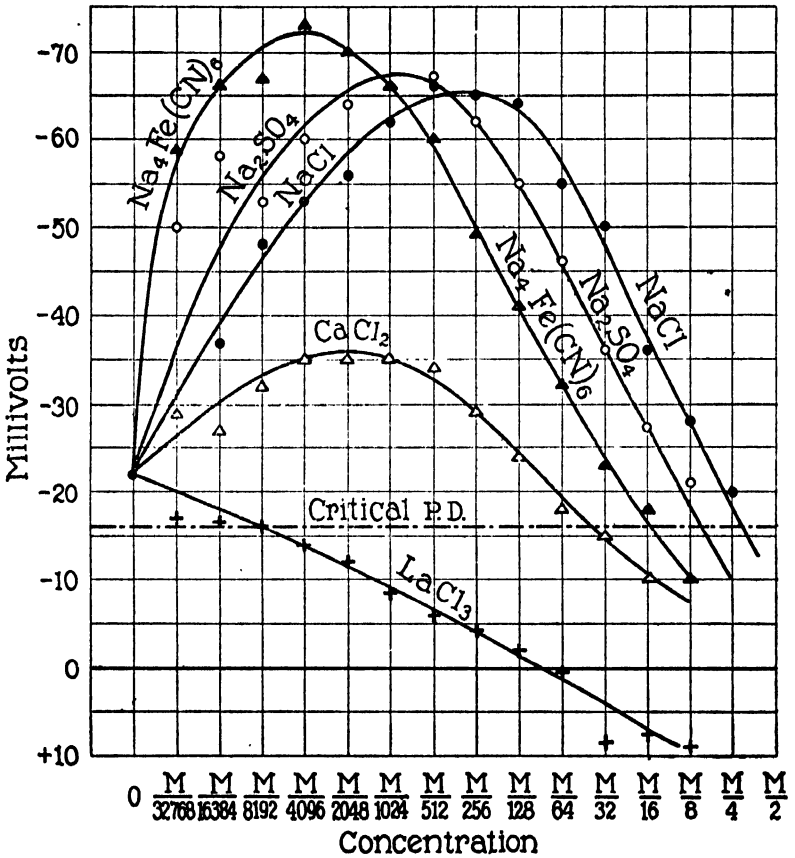

FIG. 2. Influence of $Na_4Fe(CN)_6$, Na_2SO_4, NaCl, $CaCl_2$, and $LaCl_3$ on the P.D. at pH 5.8. Addition of little salt with monovalent cation raises the P.D. to about 70 millivolts and the more rapidly the higher the valency of the anion. With $CaCl_2$ only a slight rise and with $LaCl_3$ no rise occurs in the concentrations used. In concentrations above M/64 $LaCl_3$ causes a reversal of the sign of charge of the particles.

the maximal P.D. in the latter case was 36 millivolts at a concentration of M/2,048 of the salt. After the maximum was reached the curves dropped rapidly and this drop was apparently due to the cation only. A comparison of the descending branches of the curves shows that

to bring the P.D. down from the maximum (about 70 millivolts) to *e.g.* 27.5 millivolts, the following molecular concentrations of the salts were required.

$NaCl$	M/8
Na_2SO_4	M/16
$Na_4Fe(CN)_6$	slightly less than M/32
$CaCl_2$	between M/128 and M/256

This means that the depressing action of the three Na salts is almost the same for the same concentration of cations, regardless of the anion; while the depressing effect of $CaCl_2$ is between 16 and 32 times as great as that of NaCl. This leaves no doubt that the depressing effect is due to that ion which has the opposite sign of charge to that of the collodion particle (or rather to that of the film of water moving with the particle), since the particle is negatively charged. This corresponds to the Hardy rule.

$LaCl_3$ depresses the P.D. at pH 5.8, even at low concentrations, so that at a molecular concentration of M/64 the P.D. is already zero (Fig. 2). With a further rise of the concentration of $LaCl_3$ the P.D. reverses its sign; the collodion particles assuming a positive and the water a negative charge. McTaggart observed the same reversal of the sign of charge of gas bubbles by trivalent cations. The cause of this reversal lies therefore primarily, if not exclusively, in forces inherent in the water itself. According to the curves in Fig. 2, neither $CaCl_2$ nor the Na salts are able to cause a reversal of the sign of charge of the collodion particle at pH 5.8 since the curves seem to become asymptotic to the axis of abscissæ for concentrations varying from M/16 to M/2 or above. The curves in Fig. 1 show that acids do not bring about a reversal in the concentrations used in the experiment.

The question arose whether other properties of the ion except its valency contribute to the depressing effect. The writer expected a difference in the depressing effects of LiCl, NaCl, and KCl. As a matter of fact, no difference in the influence of the three salts on the cataphoretic P.D. was noticed, as Fig. 3 shows. If differences existed they were within the limit of error in these experiments. These experiments were made at a pH of 4.7. Fig. 4 shows that the influence

of salts on the cataphoretic P.D. of collodion particles is about the same at a pH of 4.7 (Fig. 4) as at a pH of 5.8 (Fig. 2).

When the experiments were made at a higher alkalinity, namely, in a N/1,000 KOH solution (Fig. 5), the P.D. was already about 60 millivolts when no salts were added and the addition of salt could only

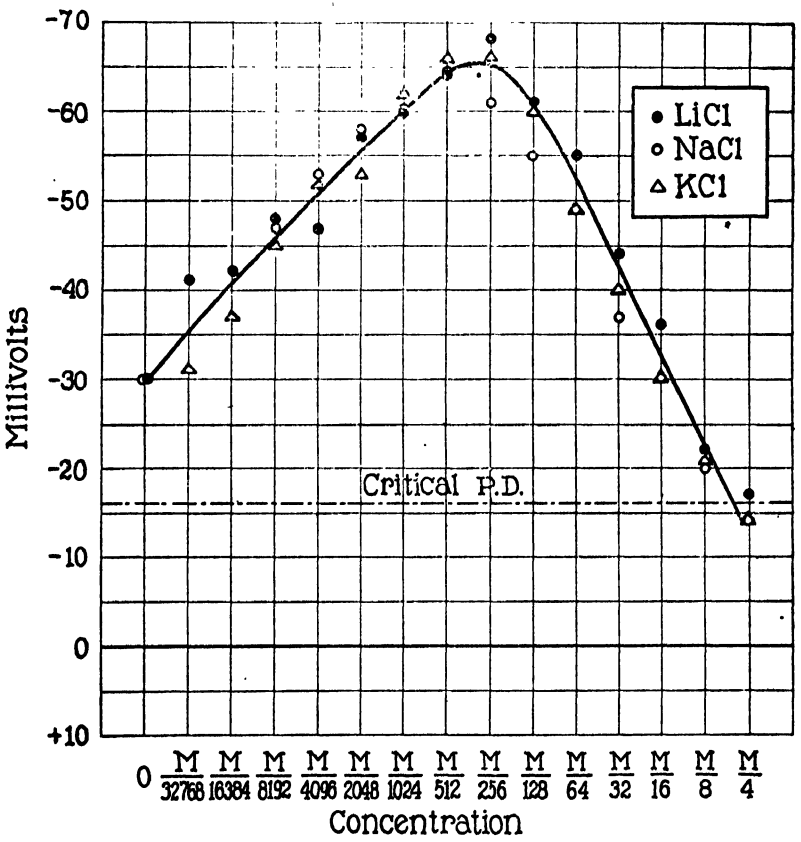

FIG. 3. Influence of LiCl, NaCl, and KCl on the P.D. at pH 4.7.

raise the P.D. to its usual maximum of about 70 millivolts. This slight rise occurred in Na_2SO_4 and $Na_4Fe(CN)_6$, but to a less amount in NaCl. In concentrations of M/256 and less, all the salts had only a depressing effect.

To bring the P.D. down to 35 millivolts in N/1,000 KOH, an approximate concentration of M/16 NaCl, of M/32 Na_2SO_4, of a little less

than M/64 $Na_4Fe(CN)_6$, and of approximately M/1,500 $CaCl_2$ was required. The depressing ion is therefore again the cation as was to be expected, since the collodion particle or rather the water film moving with it is negatively charged.

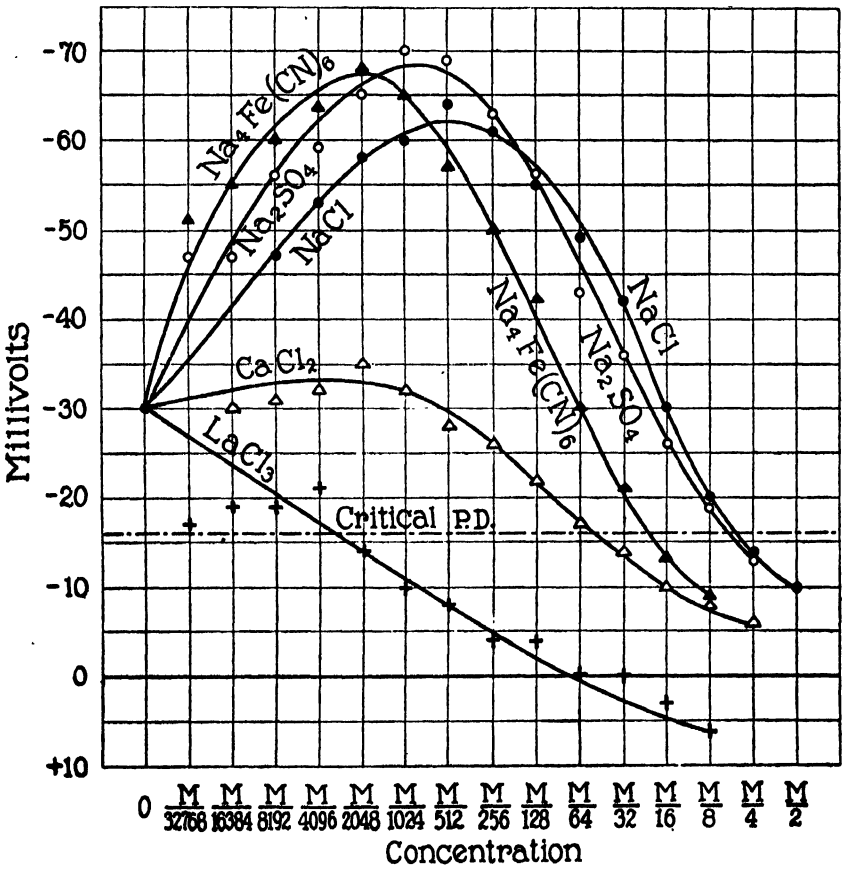

FIG. 4. Similar to Fig. 2 except that the pH was 4.7.

When the experiments were made at higher hydrogen ion concentrations, *e.g.* in N/1,000 HCl (Fig. 6), the particles had nearly their maximal charge without the addition of salt, since the P.D. was about 64 millivolts. Hence the addition of NaCl has no augmenting effect while Na_2SO_4 has a slight augmenting effect to 72 millivolts. At concentrations below M/256 the salts depress the charge of the par-

ticles. Since the particles are always negatively charged the depressing effect increases markedly with the increasing valency of the cation, as was to be expected. To depress the charge to 27.5 millivolts, a

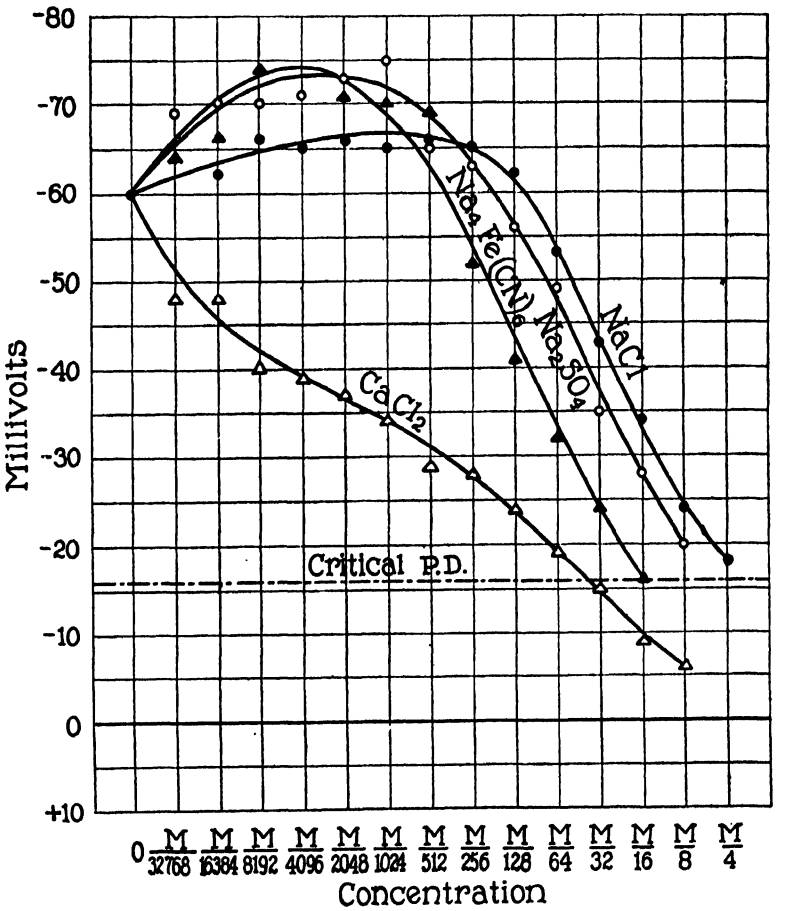

FIG. 5. Influence of salts on the cataphoretic P.D. at pH 11.0. Without salt the P.D. was already near the maximum and hence the addition of salt had only a slight augmenting effect on the P.D.

concentration of M/16 NaCl, M/256 $CaCl_2$, and M/16,384 $LaCl_3$ is required. In this acid solution $LaCl_3$ diminished the P.D. but did not bring about a reversal of the charge; perhaps for the reason that the original P.D. due to the acid was too high at the beginning.

It is often stated that H and OH ions have a greater effect on the P.D. than other ions. This was not the case in our experiments. A comparison of Fig. 1 and Fig. 2 shows that HCl, H_2SO_4, and NaOH act very much like NaCl or Na_2SO_4 on the P.D. The reason that in N/1,000 HCl or NaOH the addition of a salt no longer raises the P.D.

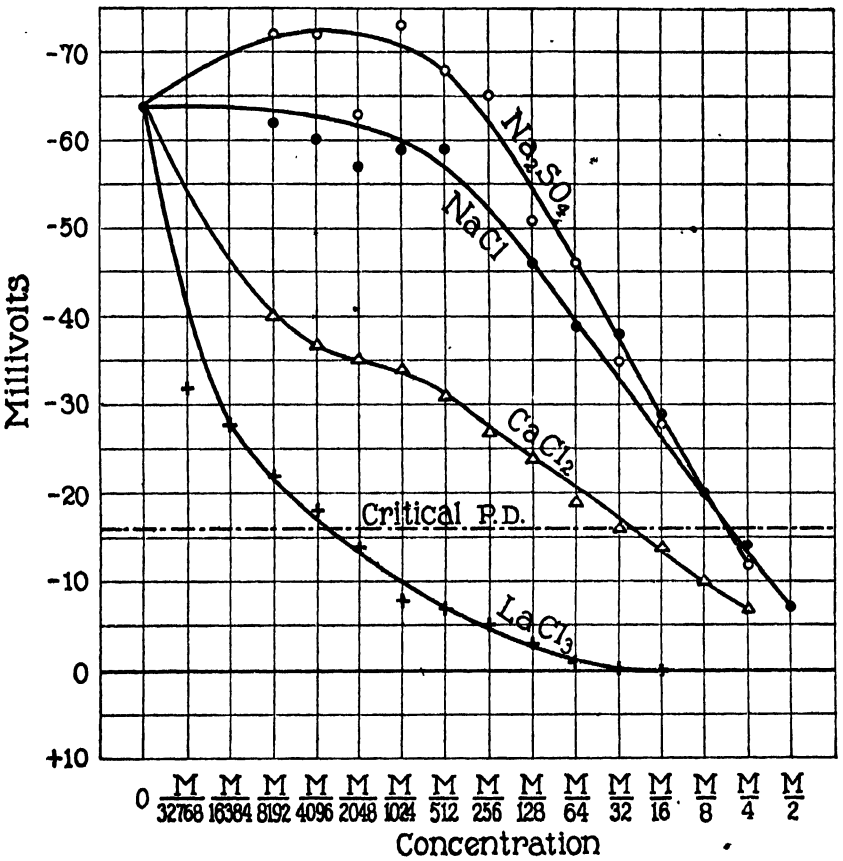

FIG. 6. Influence of salts on the P.D. at pH 3.0. (See legend of Fig. 5.)

is because the P.D. is already near the maximum before the salt is added, so that only the depressing effect of the salt becomes noticeable.

The idea that H and OH ions act more strongly than other monovalent ions is probably based on experiments with amphoteric electrolytes (where acids and alkalies bring about differences in ionization), but is apparently not true in the case of chemically inert sub-

stances like collodion. The statement that H ions act like Na ions on the cataphoretic P.D. of collodion particles is also borne out by the fact that acids do not depress the P.D. more than do Na ions, as a comparison of Figs. 1 and 2 shows. A reversal of the P.D. was observed when $LaCl_3$ was added but not when HCl or NaCl were added to the solution.

All these experiments prove, however, that it is necessary to measure the hydrogen ion concentration of the solution since otherwise the results are not strictly reproducible and comparable.

III.

The Critical P.D. *for the Precipitation of Collodion Particles from Aqueous Solutions.*

The question arose whether this cataphoretic P.D. or some other P.D. was responsible for the stability of suspensions of collodion particles in water. It can be shown that the stability of a suspension of collodion particles free from protein depends on the cataphoretic P.D., since precipitation always occurs below the same critical cataphoretic P.D. of about 16 millivolts.

When the stock suspension of collodion particles was shaken up to produce an equal distribution of particles and 1 drop of this suspension was added to 10 cc. of distilled water (of pH 5.8), the new suspension was milky when shaken up and remained so for several days. During this time the larger particles all settled and only a cloudy gray suspension was left, which gradually, after the still further settling of larger particles, gave way to a bluish opalescent suspension which lasted for many weeks ("permanently"). In this case the settling was a slow process. When 1 drop of the stock suspension was put into 10 cc. of an aqueous salt solution (also of pH 5.8) it was noticed that there existed a critical concentration of the salt, varying according to the nature of the salt, below which the suspension behaved as it did in distilled water, while in the next higher concentration a rapid, complete settling of the whole mass of collodion occurred in 12 hours or less, leaving not an opalescent but an entirely clear aqueous solution.

It was therefore comparatively easy to determine at which concentration the slow settling was replaced by a rapid settling caused by

coalescence of the small particles into larger ones. It was found that at the concentrations of salts where the rapid settling occurred, the cataphoretic P.D. between particles and aqueous solution fell below the value of about 16 millivolts; regardless of the nature of the electrolyte used for precipitation. When the P.D. was above this value, the suspension was as stable as if no electrolyte had been added; and the stability was no greater at a P.D. of 60, or 70 millivolts, than at a P.D. of 50, or 25 millivolts.

The existence of such a critical P.D. for the precipitation of suspensions agrees with the observations of Powis[7] as well as of Northrop and De Kruif.[8] Powis has made it probable that the stability of oil emulsions in aqueous solutions is destroyed when the cataphoretic P.D. between oil droplets and water is depressed below the critical value of about 30 millivolts. Northrop and De Kruif have shown that certain bacteria agglutinate when their cataphoretic P.D. is depressed by electrolytes below a critical value of 15 millivolts.

Table I gives the results of experiments on the precipitation of suspended particles of collodion by electrolytes. In one series of experiments the pH was 5.8, in a second 11.0, and in a third 3.0. In the second column of Table I are given the minimal concentrations at which precipitation was observed, *i.e.*, in which the solution became completely clear in less than 18 hours, at about 20°C., while in the fourth column are found the maximal concentrations at which the suspensions remained "permanently" stable, *i.e.* opaque for days and opalescent for weeks; in other words, where the salt caused no coalescence of particles. No attempt was made to locate the critical concentration more accurately than within the limits of concentrations given in the table, since it would probably not have been of any use in an attempt to define more sharply the real quantity of importance; namely, the critical P.D. between particles and solution. In Column 3 are found the P.D. between particles and solution at the minimal concentrations where precipitation occurred, and in the fifth column are found the P.D. of the maximal concentrations where the suspension remained stable.

[7] Powis, F., *Z. physik. Chem.*, 1914–15, lxxxix, 186.

[8] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639.

It may be pointed out that the precipitating action of acids like HCl or H_2SO_4 is of the same order of magnitude as that of Na salts but not of the order of magnitude of La salts. This agrees with the

TABLE I.

Cataphoretic Charge and Stability of Suspensions of Particles of Collodion.

1.	2. Minimum concentration required for precipitation.	3. P.D. in millivolts.	4. Maximal concentration at which suspension remains stable.	5. P.D. in millivolts.
		pH 5.8		
LiCl	M/2	(10)	M/4	17
NaCl	M/2	10	M/4	14
KCl	M/4	14	M/8	21
Na_2SO_4	M/4	13	M/8	19
$Na_4Fe(CN)_6$	M/16	13	M/32	21
$MgCl_2$	M/16	11	M/32	15
$MgSO_4$	M/16	15	M/32	19
$CaCl_2$	M/32	14	M/64	17
$LaCl_3$	M/2,048	14	M/4,096	21
		pH 11.0		
NaCl	M/2		M/4	18
Na_2SO_4	M/4		M/8	20
$Na_4Fe(CN)_6$	M/16	16	M/32	24
$CaCl_2$	M/32	15	M/64	19
		pH 3.0		
NaCl	M/2	7	M/4	14
Na_2SO_4	M/4	12	M/8	(Lost.)
$CaCl_2$	M/32	16	M/64	19
$LaCl_3$	M/2,048	14	M/4,096	18
H_2SO_4	M/4		M/8	14

statement made in an earlier part of the paper that the acids act like salts with monovalent cation (*e.g.*, NaCl) on the P.D.

The average of all the P.D. values for the minimal concentrations at which precipitation occurred was 13 millivolts, while the average of all the P.D. at the concentrations at which the suspensions remained stable was 18.5 regardless of the pH. This suggests as the probable

critical value for the P.D. where precipitation commences about 16 millivolts. The actual P.D. evaluated from the mobility by cataphoresis are probably accurate only within ± 2 millivolts of this value, which explains some of the slight deviations from this value in Table I.

These measurements confirm the conclusion reached by Powis as well as by Northrop and De Kruif that there exists a critical P.D. for the stability of suspensions, this critical P.D. being about 16 millivolts for collodion particles in aqueous solutions. When the P.D. falls below this value, the particles upon colliding are no longer repelled electrostatically but may adhere to each other and coalesce (*i.e.* agglutinate or coagulate) into larger particles which rapidly sink to the bottom of the test-tube. This coalescence of the colliding particles is due to forces of attraction between certain chemical groups of their molecules. If the P.D. is larger than 16 millivolts the particles will repel each other upon colliding with sufficient force to prevent coalescence. If this critical value is once exceeded the stability of the suspension is not increased when the charge is increased. I have noticed that there is no difference in the rate of settling of a suspension of the collodion particles when the charge varies between 20 and 70 millivolts.

Since the collodion particles are generally negatively charged, it was to be expected that only the cation of the salt should be responsible for the precipitation. This is corroborated by the fact that the precipitating efficiency of salts increases rapidly with the valency of the cation. Thus for NaCl, $CaCl_2$, and $LaCl_3$ the precipitating efficiency measured by the reciprocal value of the minimal concentration required for precipitation (Column 2, Table I) is as 1:16:1,024. This valency effect is considerably greater than it would be if the P.D. responsible for the stability were due to the Donnan effect.

The question has often been raised whether that ion of a salt which has the same sign of charge as the colloidal particle will not counteract the precipitating action of the other ion. The molecular precipitating concentrations for NaCl, Na_2SO_4, and $Na_4Fe(CN)_6$ are M/2, M/4, and about M/16 respectively. In M/2 NaCl and M/4 Na_2SO_4 the concentration of cations is practically identical. If the anion had an inhibiting effect on precipitation, the concentration of Na_2SO_4

required for precipitation should be higher than M/4 which is not the case. The precipitating concentration of $Na_4Fe(CN)_6$ is even lower than that to be expected if only the Na ion acted.

It follows from this that the force preventing coalescence of the colloidal particles into larger aggregates which settle rapidly is the *cataphoretic* and no other potential difference between the particles and the aqueous solution, and that there is a critical value for this P.D. which in the case of collodion particles is about 16 millivolts. As soon as the cataphoretic P.D. falls below this value the collodion particles will coalesce. The depressing and precipitating action of a salt on the negatively charged collodion particles depends practically exclusively on the cation of the salt and increases rapidly with the increase in the valency of the cation. There exists no pep'tization effect of plurivalent anions.

SUMMARY AND CONCLUSIONS.

1. When collodion particles suspended in water move in an electric field they are, as a rule, negatively charged. The maximal cataphoretic P.D. between collodion particles and water is about 70 millivolts. This is only slightly more than the cataphoretic P.D. found by McTaggart to exist between gas bubbles and water (55 millivolts). Since in the latter case the P.D. is entirely due to forces inherent in the water itself, resulting possibly in an excess of OH ions in the layer of water in contact and moving with the gas bubble, it is assumed that the negative charge of the collodion particles is also chiefly due to the same cause; the collodion particles being apparently only responsible for the slight difference in maximal P.D. of water-gas and water-collodion surfaces.

2. The cataphoretic charge of collodion particles seems to be a minimum in pure water, increasing as a rule with the addition of electrolytes, especially if the cation of the electrolyte is monovalent, until a maximal P.D. is reached. A further increase in the concentration of the electrolyte depresses the P.D. again. There is little difference in the action of HCl, NaOH, and NaCl or LiCl or KCl.

3. The increase in P.D. between collodion particles and water upon the addition of electrolyte is the more rapid the higher the valency

of the anion. This suggests that this increase of negative charge of the collodion particle is due to the anions of the electrolyte gathering in excess in the layer of water nearest to the collodion particles, while the adjoining aqueous layer has an excess of cations.

4. In the case of chlorides and at a pH of about 5.0 the maximal P.D. between collodion particles and water is about 70 millivolts, when the cation of the electrolyte present is monovalent (H, Li, Na, K); when the cation of the electrolyte is bivalent (Mg, Ca), the maximal P.D. is about 35 to 40 millivolts; and when the cation is trivalent (La) the maximal P.D. is lower, probably little more than 20 millivolts.

5. A reversal in the sign of charge of the collodion particles could be brought about by $LaCl_3$ but not by acid.

6. These results on the influence of electrolytes on the cataphoretic P.D. between collodion particles and water are also of significance for the theory of electrical endosmose and anomalous osmosis through collodion membranes; since the cataphoretic P.D. is probably identical with the P.D. between water and collodion inside the pores of a collodion membrane through which the water diffuses.

7. The cataphoretic P.D. between collodion particles and water determines the stability of suspensions of collodion particles in water, since rapid precipitation occurs when this P.D. falls below a critical value of about 16 millivolts, regardless of the nature of the electrolyte by which the P.D. is depressed. No peptization effect of plurivalent anions was noticed.

The mobility measurements required for the determination of the P.D. were made by Mr. M. Kunitz.

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EXPERIMENTAL STUDIES IN DIABETES.

SERIES II. THE INTERNAL PANCREATIC FUNCTION IN RELATION TO BODY MASS AND METABOLISM.*

10. THE INFLUENCE OF THE THYROID UPON DIABETES.

BY FREDERICK M. ALLEN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

The writer previously[1] reviewed the literature of this subject up to 1913. This literature comprised some of the early clinical reports of the association of glycosuria or diabetes with hyperthyroidism, and the production of glycosuria in normal persons by overdosage with thyroid preparations; also the high carbohydrate tolerance of most cases of myxedema or cretinism, and the lowering of this tolerance by thyroid treatment. Experimentally thyroidectomy with preservation of the parathyroids was stated by various authors to raise the sugar tolerance of normal animals and to reduce the glycosuria following pancreatectomy or epinephrin injections. Histologic changes in the thyroid following removal of the pancreas or in the pancreas following removal of the thyroid have been reported without convincing demonstration. The facts, as far as valid, were all open to a simple criticism of interpretation, namely, that though glycosuria might be partially suppressed by the metabolic injury of thyroidectomy, no evidence had ever been brought to indicate any actual benefit in the form of restoration of the power of utilizing sugar; on the contrary, diabetic symptoms were merely replaced by cachexia and the animals died more quickly with than without thyroidectomy.

* The first four papers of this series were published in the *American Journal of the Medical Sciences*, Vol. 160, 1920, p. 781, and Vol. 161, 1921, pp. 16, 165, and 350. Papers 5 to 9 were published in the *American Journal of Physiology*, Vol. 54, 1920–1921, pp. 375, 382, 425, 439, and 451. Papers 11 and 12, completing the series, will be published in subsequent issues of the JOURNAL OF METABOLIC RESEARCH.

A considerable literature which has accumulated since that time may be partially reviewed under the following heads:

EXPERIMENTAL: A. *Thyroid administration in normal animals.*
B. *Thyroidectomy in normal animals.*
C. *Thyroidectomy in diabetic animals.*

CLINICAL: A. *Carbohydrate metabolism in thyroid disorders.*
B. *Association of thyroid disorders with diabetes.*
C. *Thyroid operations in diabetic patients.*

EXPERIMENTAL.

A. *Thyroid Administration in Normal Animals.*

The earlier attempts with thyroid feeding of normal animals gave varying results. Some features of clinical hyperthyroidism have thus been reproduced in the successful cases, but a more accurate and effective means has been afforded by Kendall's[2] discovery of thyroxin. Its injection into dogs, goats and other species has produced toxic symptoms and increased metabolic rate. According to Plummer,[3] the condition of human patients with thyroid adenoma and accompanying simple excess of thyroid secretion is thus imitated. Genuine exophthalmic goitre has not been produced in animals by feeding or injection of normal thyroid substance or extract. Doubt exists concerning claims, such as that of Klose, Lampé and Liesegang,[4] that the injection of goitre juice from human exophthalmic cases reproduces in animals the entire picture, including exophthalmos, nervous symptoms, tachycardia, elevated temperature, sweating, falling hair, hyperglycemia and glycosuria.

Cramer and collaborators[5a] demonstrated that thyroid feeding lowers the glucose tolerance of normal dogs, also[5b] that it causes complete or almost complete loss of liver glycogen in cats and rats, even when the animals are kept on carbohydrate-rich diet. No glycosuria occurs, and the respiratory quotient shows a high and

prolonged elevation, indicating a very active combustion of carbohydrate. They conclude that the thyroid inhibits the formation of glycogen; also, that deficiency of glycogen formation is thus excluded as an explanation of glycosuria. Parhon[6] found that thyroid feeding of rabbits reduced the liver glycogen as low as one-sixth of the quantity found in control animals, but did not cause it to disappear. The muscle glycogen was not reduced, and the respiratory quotient held the high level characteristic of carbohydrate diet. The suggestion was offered that the deficiency of liver glycogen may have been due to the muscular activity of the thyroid-fed animals, which were very nervous, with trembling legs and ears. Kuriyama,[7] feeding large quantities of fresh thyroid to rabbits and rats, found a marked reduction in liver glycogen, which was independent of diet, and which persisted after such parenteral doses of glucose as quickly increased the glycogen of normal controls. Neither species showed glycosuria or hyperglycemia from the thyroid dosage, and the tolerance of rabbits for glucose parenterally was not lowered. The sensitiveness to glycosuria and hyperglycemia from epinephrin was also not appreciably changed. The epinephrin content of the adrenals of thyroid-fed rats was not altered. Horrisberger[8] proved that thyroid feeding increased the metabolism of phlorizinized white rats in the same manner and degree as in controls without phlorizin, thus opposing the view that the metabolic increase is due to lack of the sparing action of carbohydrate. Abelin and Jaffé[9] discovered that phenylethylamin and tyramin behave similarly to thyroid extract in increasing total metabolism, reducing liver glycogen and raising the respiratory quotient.

Kojima[10] described enlargement of the pancreas and mitoses and other changes in its acinar cells in thyroid-fed rats, and these findings were confirmed by Hoshimoto.[11] An explanation is difficult, as Kojima still obtained these results when the thyroid extract was boiled. He found no changes in the islands, and there are no reliable reports of changes in them from thyroid administration.

B. *Thyroidectomy in Normal Animals.*

No apparent disturbance of health follows thyroidectomy in adult dogs, at least for several months after the operation. The acute

death formerly considered as the rule is now known to be due only to loss of the parathyroids. The sparing of two or more parathyroids is considered a safeguard against any recognizable parathyroid deficiency. The removal of the thyroid alone, however, causes abnormalities of development in puppies; also, the fatal cachexia described by earlier authors[12] many months after thyroidectomy in adult dogs seems not explainable by any known parathyroid defect, and a doubt is thus raised whether dogs can actually live indefinitely without thyroids. Eppinger, Falta and Rudinger[13] discovered that the nitrogen excretion of a fasting thyroidectomized dog quickly falls to a lower level than that of a normal fasting animal, and this level cannot be further reduced by fat or carbohydrate feeding. Their report that the excessive nitrogen excretion of depancreatized dogs is reduced by thyroidectomy was corroborated by Lusk[14] with a similar observation in phlorizin glycosuria.

The place assigned to the thyroid by Eppinger, Falta and Rudinger and their followers in their pluriglandular speculations has been sufficiently discussed heretofore.[1] Among their claims were assertions that thyroidectomy raises the tolerance of animals for test doses of glucose, prevents glycosuria from epinephrin or the Bernard puncture, and appreciably mitigates the effects of pancreatectomy. Lorand[15] had previously alleged that thyroidectomy two days after pancreatectomy abolishes the glycosuria; also that pancreatectomy is followed by signs of over-function of the thyroid, and that thyroidectomy gives rise to hypertrophy of the islands of Langerhans This finding regarding islands was supposedly confirmed by Falta and Bertelli.[16] The present writer had the opportunity of studying the pancreas of one of Janney's dogs which had been thyroidectomized several months previously, and is convinced of the absence of any variation from the normal structure.

Excluding early experiments in which parathyroids were removed along with thyroids, the increase of glucose tolerance in thyroidectomized animals has in general been confirmed by later writers. McCurdy[17] observed such an increase, using the Blumenthal method of single intravenous injections, thus ruling out the factor of intestinal absorption but not the possibility of an altered renal threshold. McLean[18] stated that the hearts of thyroidectomized rabbits utilized

less glucose than those of normal rabbits in perfusion experiments by the Locke method, but as only a brief preliminary report was published without sufficient record of details, and as the method employed was so uncertain and the results so contradictory to those of others, this work may be dismissed as inaccurate or misleading. Janney and Isaacson[19] found that hypoglycemia is the rule in thyroidectomized dogs, and that the rise of blood sugar after administration of glucose by stomach is delayed as compared with normal dogs. The percentile rise, in comparison with the preceding hypoglycemia, is greater than in normal animals, but the absolute level of blood sugar remains lower than in the normal controls. The question of delayed alimentary absorption was tested by feeding meat and demonstrating practically identical rates of nitrogen excretion before and after thyroidectomy.

The assertion that epinephrin injections produce no glycosuria in thyroidectomized animals was disproved by Underhill.[20] Böe[21] found the same hyperglycemia from adrenalin injections in rabbits before and after thyroidectomy. The statement of some authors that the effect of adrenalin is less after thyroidectomy is open to question, because of the variable results of different injections, particularly after repeated doses in the same animal.[22]

With reference to the cessation of glycosuria in depancreatized dogs after thyroidectomy as reported by Lorand,[15] notice must be taken of the fact that the animals were not benefited but their lives were apparently shortened. Similarly MacCallum[23] performed this experiment upon two diabetic dogs. In one the pancreatectomy was not quite complete; glycosuria ceased after thyroidectomy and the animal died in 3 days. The completely depancreatized dog showed merely some reduction of glycosuria after thyroidectomy and died the following day. In the experience of Massaglia,[24] thyroidectomy performed simultaneously with pancreatectomy did not alter the glycosuria, but when the pancreatectomy was performed several days after the thyroidectomy the sugar excretion was lower than usual. Friedman and Gottesman[25] have described disappearance of glycosuria in both partially and totally depancreatized dogs after thyroidectomy. Some of the animals lived for several weeks, but a sufficient demonstration of actual benefit from the thyroidectomy is

lacking. The reduction of nitrogen excretion in normal, depancreatized and phlorizinized dogs alike, as described by Eppinger, Falta and Rudinger and by Lusk, is best interpreted in the sense of a specific influence of the thyroid upon protein metabolism, rather than as an amelioration of the diabetes or evidence of an antagonism between the thyroid and the pancreas.

CLINICAL.

A. *Carbohydrate Metabolism in Thyroid Disorders.*

The most numerous contributions under this head consist of studies of the carbohydrate metabolism by the aid of glucose tolerance tests and blood sugar analyses. It will suffice to mention a series of representative findings in chronological order.

Flesch[26] performed 65 tests on 40 patients with Basedow's disease. None of them showed fasting or spontaneous hyperglycemia. After the giving of 100 gm. glucose in tea, 17 out of 28 patients had blood sugar curves above those of normal persons. After partial thyroidectomy, in the early period a still higher number, namely 19 out of 28 reacted with abnormal hyperglycemia, but after passage of a longer time a distinct lowering of the curves was found. Two myxedema patients reacted to the glucose with hyperglycemia equal to that of the hyperthyroid cases. They were treated by implantation of healthy human thyroid tissue, and subsequently exhibited still greater hyperglycemia.

Schulze[27] observed glycosuria in 4 out of 16 hyperthyroid patients after ingestion of 100 gm. of glucose. The susceptibility to alimentary glycosuria was found to run parallel with a similar susceptibility to adrenalin glycosuria. The quantities of sugar excreted were always small. The longest and severest cases of hyperthyroidism were the most readily subject to glycosuria. Both glycosuria and hyperglycemia were diminished by partial thyroidectomy.

Geyelin[28] found some degree of hyperglycemia (defined as blood sugar above 0.1 per cent.) in 90 per cent. of moderate or severe cases of hyperthyroidism. Glycosuria, either spontaneously or after administration of 100 gm. glucose, was also common. Thyroid treatment raised the blood sugar curve of myxedema patients.

Du Bois[29] studied the respiratory metabolism of 11 patients with exophthalmic goitre and 1 cretin. Notwithstanding increased basal metabolism as high as 50 to 75 per cent. above normal, the specific dynamic action of both protein and glucose was within normal limits. The respiratory quotient rose sharply after glucose ingestion, indicating active carbohydrate combustion even in the presence of glycosuria. One patient derived 89 per cent. of his total energy from carbohydrate while excreting sugar in the urine. The conclusion is drawn that the glycosuria in such cases must be explained by abnormal mobilization and not deficient utilization of carbohydrate.

Woodyatt, Sansum and Wilder,[30] also Wilder and Sansum, using the method of continuous intravenous glucose injections at fixed rates, confirmed the reduced sugar tolerance in hyperthyroidism. As compared with the normal assimilative capacity of about 0.85 gm. of glucose per kilogram per hour, the tolerance in exophthalmic goitre was found to be between 0.5 and 0.7 gm., according to the severity. This reduction of tolerance was regarded as only apparent, due to the fact that the hyperglycogenolysis of hyperthyroidism furnishes an extra supply of endogenous sugar to the organism. The findings contradicted the current belief in an increased sugar tolerance with hypothyroidism. A myxedema patient was found to assimilate glucose only at the normal rate of 0.85 gm. per kilogram per hour. Similarly, patients with hypopituitarism were found able to utilize glucose only at the normal rate, even when the highest dosage by stomach failed to produce glycosuria, the explanation being found in retarded intestinal absorption.

Janney and Isaacson[19] found low blood sugar curves in hypothyroid patients in glucose tolerance tests. In hyperthyroidism the results were mixed; by no means all the curves were high, and the majority were not above normal. A delayed rise of the blood sugar curve was however, found general in both hypothyroid and hyperthyroid cases. In the latter, the rise was more apt to be superposed upon a high or normal blood sugar level, and in the former it was more apt to be superposed upon an originally subnormal level, thus giving the characteristic peculiarities. Janney and Henderson[19] likewise obtained mixed results, less constant than the findings in dogs.

Denis and Aub[31] found fasting hyperglycemia extremely rare in their cases of hyperthyroidism. An excessive rise of blood sugar after ingestion of 100 gm. glucose and 50 gm. bread was found in every case examined. No regular relation was demonstrable between hyperglycemia and glycosuria on the one hand and the severity of the toxic condition on the other. Improvement of the condition by rest or operation was sometimes accompanied by reduction of the tendency to hyperglycemia. In two cases of hypothyroidism no change in the fasting blood sugar was observed to result from administration of thyroid extract.

Hamman and Hirschman[32] reported abnormal hyperglycemia with glucose tolerance tests in hyperthyroid cases, and the curves were often slow in reaching their maximum. In one instance a normal curve was found one month after partial thyroidectomy. Epinephrin caused a high and prolonged rise of blood sugar in hyperthyroid cases.

Lueders[33] found high and prolonged elevation of blood sugar to be the rule in glucose tolerance tests in hyperthyroidism, but the results were not constant.

Sanger[34] studied 8 cases of Graves' disease in which the basal metabolism was 30 per cent. or more above normal. After a fast of 14 to 16 hours, each patient drank a glucose solution which generally represented 1.75 gm. per kilogram of body weight. Blood sugar analyses and respiration determinations with the Tissot apparatus were performed at short intervals. Starting with fasting respiratory quotients somewhat lower than those of the normal controls, the hyperthyroid patients exhibited as their most striking characteristic a very rapid rise to a high quotient, approaching or even exceeding 1, and the maintenance of this high level through the 2½ hours of observation, while the quotients of the normal control subjects rarely rose above 0.9 and tended to fall slightly before the close of the experiments. One mild case showed little alteration of glucose tolerance; but the characteristic hyperglycemic response of hyperthyroidism was present in all of the 7 more severe cases, and 6 of these showed glycosuria. It was concluded that the specific dynamic action of carbohydrate was probably the same in the hyperthyroid cases as in the normal controls, and that the hyperglycemia and lowered tolerance of Graves'

disease is due not to inability to utilize carbohydrate but probably to a decreased ability of the liver to store it. Both the hyperglycemia and the exaggerated rate of sugar combustion were interpreted in harmony with Cramer's observations of lack of glycogen formation in the liver with thyroid intoxication.

Morris[35] found the glucose tolerance test of confirmative value in the diagnosis of mild or doubtful cases of hyperthyroidism. He found that the blood sugar in the hyperthyroid cases rose higher than normal and returned to a normal level in about 4 hours; the maximal hyperglycemia was reached in 1½ to 2½ hours, while in normal controls it was reached within 1 hour.

Boothby[36] concluded from a similar study that "the blood sugar curves following the ingestion of 100 gm. of glucose have not been sufficiently consistent in the different types of cases studied to be of diagnostic value, in spite of the fact that high and prolonged curves were more frequently found in patients with hyperthyroidism than in those with hypothyroidism."

Olmstead and Gay,[37] using glucose tolerance tests, found a distinction between the hyperglycemic curves of hyperthyroidism and of diabetes, in that the former were high but steep, while the latter were both higher and more prolonged.

B. *Association of Thyroid Disorders with Diabetes.*

Chvostek[38] reported alimentary glycosuria in 60 per cent. of his cases of Basedow's disease. Kocher,[39] somewhat later, also found the combination frequent. It is difficult to determine what proportion among the cases of glycosuria represented actual diabetes, but the latter is evidently rare. The literature of this association is reviewed by Labbé,[40] who states that Gastand in a thesis in 1913 collected a total of 58 instances reported by authors up to that time, and by Fitz,[41] who mentions that Billings observed only 1 case of glycosuria among 61 cases of exophthalmic goitre, and that Greeley[42] found only 6 instances of exophthalmic goitre among 614 diabetics of Waukesha. Labbé, described 5 cases of the combination from his own experience, but doubted the rôle of the thyroid in the etiology of the diabetes. Fitz gave an account of 39 previously unreported examples

of the combination, 33 of them from the Mayo Clinic and 6 from the Massachusetts General Hospital. As evidence of the rarity, he stated that only 9 cases of diabetes were found among 1800 cases of exophthalmic goitre in the Mayo Clinic. Because of this reason, and the fact that the diabetes might either precede or follow the thyroid trouble, he concluded that the association is fortuitous. The patients with non-toxic goitre showed no improvement in their diabetes after partial thyroidectomy. Certain patients, on the other hand, with toxic thyroid disease and diabetes, improved considerably in respect to their diabetes after the thyroid intoxication was relieved. This benefit was believed to be sufficiently explained by a reduction of the metabolic rate, acting similarly to a reduction of diet. Some further references to hyperthyroidism in association with diabetes are contained in the paper of Rohdenburg, mentioned below.

According to the testimony of a series of authors, the administration of thyroid substance may not only cause glycosuria in normal persons and reduce the usually high carbohydrate tolerance of hypothyroid patients, but may also stand in suspicious relationship with the onset of true diabetes. Friedrich Müller[43] gave thyroid tablets for several weeks to a woman with Basedow's disease. The Basedow symptoms were aggravated, and sugar appeared in the urine in increasing quantities. The glycosuria persisted after the tablets were discontinued; the patient died several months later in diabetic coma. The occurrence of glycosuria in several normal persons after large dosage with thyroid tablets was also recorded by Müller. He further mentioned a physician's wife who had a large goitre for some years and was treated with thyroid tablets. During these years she had continuous glycosuria of 3 to 5 per cent, but it was not known whether this antedated the thyroid treatment or not. In later years the goitre decreased in size and thyroid treatment was stopped, and glycosuria thereafter remained absent, even after eating of the largest quantities of carbohydrate.

Strasser[44] described a cretin 8 years of age, treated by thyroid feeding beginning in July, and in September polyphagia, thirst, polyuria and glycosuria were present. The thyroid tablets were omitted and diet and codein were tried, but the diabetes went on to termination 17 months later in coma.

One case of diabetes developing after treatment of myxedema by thyroid feeding was seen by Allen, Stillman and Fitz,[45] but study of this case proved that doses of thyroid suitable for treatment of the myxedema were entirely compatible with improvement of the food tolerance, and it seemed probable that the myxedema and the diabetes were independent disorders. Other reports of hypothyroidism associated with diabetes were referred to in the writer's former review.[1]

C. *Thyroid Operations in Diabetic Patients.*

In one sense, Falta's[46] trial of X-ray treatment of the thyroids of six exophthalmic goitre patients may be considered as the beginning of these procedures. Four of these had diabetes, the others only more or less fatty indigestion as a complication. In Case I (diabetes with steatorrhea), irradiation of the goitre was without effect, and death occurred from diabetes and pyelonephritis. Various autopsy findings are mentioned, but nothing is said of any examination of the pancreas. Case II represented diabetes without fatty stools; irradiation of the goitre was again fruitless. In Case III the diabetes apparently antedated the hyperthyroidism. The slight glycosuria ceased after the second Roentgen treatment of the thyroid. In the fourth instance (Case VI) irradiation of the goitre supposedly cured the diabetes, but the case had been extremely mild from the outset. In the remaining 2 cases the X-ray applied to the thyroid supposedly raised the sugar tolerance. In view of the uncertain efficacy of Roentgenotherapy of the thyroid, and the known influence of hospital care and moderate dietary restriction upon both thyroid disease and mild diabetes, the above evidence must be considered inadequate for establishing any conclusions. In addition, it must be recognized that the combinations represented in these few cases are not typical but are actually rare.

More striking observations in two still more unusual cases have been published by Rohdenburg.[47] One of these occurred in a family with a high hereditary incidence of diabetes. In this connection a chart was shown, illustrating marked increase of diabetic glycosuria from the feeding of either desiccated thyroid or desiccated adrenal glands. After the deaths of three members of this family from

diabetes, the son of one of them, who had been diabetic for some time previously, developed exophthalmic goitre. He then disappeared from observation. After five years he was seen again, and gave a history of complete freedom from diabetic symptoms in consequence of a partial thyroidectomy. Diet restrictions had been discarded, and the author proved that "the consumption of two pounds of grapes and three ice-cream sodas within 12 hours at the time of his visit failed to produce sugar in the urine." The second case reported was that of a patient with exophthalmic goitre, whose father had died of diabetes. One lobe of the thyroid had been removed, with resultant general improvement and gain of 15 pounds' weight. Mild diabetes subsequently developed (glycosuria 1.8 to 2.5 per cent., blood sugar 140 mg. per 100 cc., cessation of glycosuria within 16 hours of fasting). After 5 days of sugar-freedom, the other thyroid lobe was extirpated, leaving the isthmus. On the day after operation there was glycosuria of 4.8 per cent, which gradually disappeared in the following 4 days. Within a month after operation the patient had gained 25 pounds in weight, hyperthyroid symptoms were absent, and 2 ounces of cane sugar added to a diet consisting entirely of carbohydrate failed to produce glycosuria.

From the surgical treatment of goitre in the presence of a complicating diabetes to the partial removal of a normal thyroid in the attempt to cure diabetes is a considerable step. If Falta and collaborators had had full faith in their own doctrines, they should have adopted the measures for which Crile[48] actually gained priority some years later. He removed one adrenal and approximately three-fourths of the thyroid, and divided both cervical sympathetic trunks, in a series of patients with supposed injurious preponderance of a group of glands, including one diabetic. Two years after the first of these operations, he described the general results as encouraging. During several months which had elapsed since the operation on the man with mild diabetes, glycosuria had ceased and a high tolerance had been attained, and at least a share of these benefits was credited to the surgical treatment. In the latest book of Crile[49] on the thyroid, however, there is no mention of its influence upon carbohydrate metabolism and no advocacy of its resection in diabetes.

O'Day[50] limited his diabetic surgery to the thyroid. He first reported two cases of combined diabetes and exophthalmic goitre. One patient was a man aged 24, treated first by injection of boiling water into the thyroid and later by a partial resection. Glycosuria ceased as the symptoms of thyroid intoxication improved, and continued absent when diet was disregarded and sugar and candy eaten. The second patient was a woman with diabetes who subsequently developed exophthalmic goitre. Partial thyroidectomy was followed by acute intoxication and dangerous acidosis; then gradually all symptoms were relieved and a supposedly normal carbohydrate tolerance was attained. In a brief note, O'Day stated: "Encouraged by the experience of having several cases of glycosuria associated with exophthalmic goitre clear up when the hyperthyroidism was corrected, two cases of diabetes in young subjects with no goitre symptoms were treated by removing the greater part of the thyroid gland. The results were as follows: One was restored to complete carbohydrate tolerance, the other to a tolerance of nine ounces of carbohydrate per day." This report was published after a few months of observation of these cases, and there has been no further mention of the use of this method by O'Day or other surgeons.

SUMMARY.

It is significant that the general trend of evidence has steadily reduced the supposed rôle of the thyroid in diabetes. The loose early statements of the frequency of glycosuria with clinical hyperthyroidism have not held good. A broad rule with few if any exceptions is that toxic agents lower the apparent assimilation of carbohydrate. It is therefore to be expected that toxic goitres will be associated with more or less lowering of the assimilation, and it is only surprising that this reduction does not run parallel with the degree of intoxication, is lacking altogether in many cases, and in the great majority of cases is so slight that it does not give rise to spontaneous glycosuria and is demonstrable only by the most refined tests. Similarly, poisoning of normal persons or animals by thyroid products may be expected to depress the apparent assimilation of glucose, as judged by slight glycosuria or hyperglycemia, but it is noteworthy

that an impairment of combustion in the diabetic sense is proved by the high respiratory quotients to be absent. The poverty of liver glycogen, whether due to deficient formation or excessive consumption, was rightly interpreted by Cramer as proof that glycosuria and diabetes are not explainable by mere lack of glycogen storage. It has not yet been proved whether this scarcity of liver glycogen is specific to thyroid intoxication or may be found also in other forms of intoxication, as by bacterial toxins, associated with an equal rise of total metabolism. Both the intoxication and the elevation of metabolism may tend to aggravate any existing or latent diabetes, and relief from the intoxication may be expected to benefit the diabetes and perhaps allow it to return to its latent state. On the other hand the combination of hyperthyroidism with diabetes is so rare that it must be considered accidental; the association of diabetes with hypothyroidism is also known; and the onset of diabetes after administration of thyroid tablets is another rarity which may be ranked on a par with the formerly accepted "traumatic diabetes," namely as representing merely the outbreak or the discovery of a preexisting diabetes. The elevation of apparent glucose tolerance produced in normal animals by thyroidectomy is trivial in degree. Thyroidectomy in diabetic animals is best regarded as an additional injury which may to some extent suppress the typical mobilization of sugar; the most important point is the total lack of evidence of the recovery of any of the normal power to burn sugar. Here is another instance of the disastrous confusion between glycosuria and diabetes; as it is incomprehensible that a totally depancreatized animal should regain any of the power of normal sugar utilization which is dependent upon the pancreas, it must be impossible that the removal of any other organ could effect any amelioration of the diabetes in the true sense of the word. The treatment of clinical diabetes by the removal of organs supposedly antagonistic to the pancreas has evidently failed. Altogether, the investigations have contributed a little information concerning the specific function of the thyroid, and have furnished more complete disproof of the imagined opposition between the thyroid and pancreas, and of the hypothetical rôle of the thyroid in the etiology of diabetes, than existed at the time of the former review.

The writer's experiments in the former publication mentioned[1] consisted in (a) feeding of thyroid to normal animals, with the result of a trivial lowering of their glucose assimilation, and to partially depancreatized animals, with the result that no diabetes was produced even in animals very close to the verge of diabetes; and (b) partial thyroidectomy (removal of not more than seven-eighths of the thyroid) in diabetic animals, with negative effects upon the diabetes even when considerable portions of the adrenals were also removed. It was desired to repeat the thyroid feeding experiments upon partially depancreatized animals, in order to test the influence both upon pancreatic deficiency which was not quite sufficient to cause diabetes, and also upon the course of an existing diabetes. The previous thyroidectomy experiments had imitated any probable clinical procedures, since it is not likely that any surgeon would contemplate the removal of more than seven-eighths of the thyroid of a human diabetic; but for theoretical information it seemed desirable to make a trial of complete thyroidectomy in dogs with various degrees of diabetes. This line of experimentation might prove instructive in two directions; first in regard to any specific thyroid influence, and second in regard to a possible influence of raising or lowering the total metabolism. The latter point in particular pertains to the general subject matter of this series of papers. The observations will be presented in two groups: I., experiments with thyroid feeding; and II., experiments with thyroidectomy.

1. Experiments with Thyroid Feeding.

Dog B2-25.—Male, mongrel, normal weight 18.2 kg., was partially depancreatized on Dec. 16, 1913, so that the remnant left was estimated at 1/12 to 1/13 of the gland, as previously described.[51] He was kept free from diabetic symptoms by strict undernutrition, and the food tolerance gradually rose in the ensuing months. At first as little as 200 gm. of beef sufficed to cause glycosuria, but by November, 1914, the protein tolerance was so high that glycosuria resulted only when as much as 1400 gm. of beef lung was fed at one time, as described in another place. Beginning Nov. 27, the dog was placed for one week on a diet of 800 gm. cooked beef lung and 200 gm. raw beef thyroid. Neither glycosuria nor any symptoms of hyperthy-

roidism resulted. The tolerance and health seemed to be as good on the thyroid diet as on the same quantities of muscle or lung.

Dog B2-43.—Female, bull terrier mongrel, in consequence of two pancreas operations previously described,[52] reached a condition such that glycosuria resulted from moderate quantities of starch as shown by published tests,[53] but did not result from the largest quantities of protein that could be eaten. July 21, 1914, a regular diet of 1 kg. of beef lung was begun, the body weight being 9.7 kg. The urine remained normal except on July 28, when a single feeding of bread and soup mixture was given by mistake, with resultant heavy glycosuria. Changes of diet were then made as follows:

Date.	Body weight.	Diet.
	kg.	
Aug. 17	9.9	900 gm. beef lung and 100 gm. raw calf thyroid.
" 20	9.9	800 " " " " 200 " " " "
" 22	10.0	700 " " " " 300 " " " "
" 25	10.2	500 " " " " 500 " " " "
" 28	10.3	300 " " " " 700 " " " "
" 31	10.2	1 kg. " " "
Sept. 2	10.2	1 kg. " "

Neither glycosuria nor changes in the appearance, behavior, pulse, or general health were observed in consequence of the high thyroid feeding.

As described in the reference cited, on Sept. 10 the feeding of 50 gm. of starch in the form of oatmeal caused no glycosuria, but on Sept. 15 the same quantity of starch in the form of rice produced an excretion of 5.5 gm. of sugar.

In this experiment thyroid feeding failed to produce glycosuria in an animal which was readily subject to glycosuria from starch.

Dog B2-79.—Male, bull terrier mongrel, normal weight 15 kg., was partially depancreatized on Nov. 10, 1914, the remnant being estimated at 1/8 of the pancreas. Portions of the record have been given in former publications.[54] The glycosuria which was present on bread feeding ceased on a diet of 1 kg. of beef lung. The earlier published

experiments showed how tolerance was gradually lost as the dog was fattened. The diet of 1 kg. raw lung continued from April 7 to May 30, 1915, without glycosuria. Beginning May 30, the diet was changed to 500 gm. lung and 500 gm. fresh sheep thyroid. June 4, the diet of 1 kg. beef lung was resumed, and glycosuria, which had been absent before, appeared to the extent of 0.6 per cent. in 417 cc. of urine on this day. It then remained absent on the same diet to June 26.

On June 26, the diet was changed to 1 kg. of fresh sheep thyroid daily. On July 1 the diet of 1 kg. raw beef lung was resumed. The urine record for this period was as follows:

Date.	Urine vol.	Glycosuria.
	cc.	*per cent.*
June 26	305	0
" 27	451	0
" 28	452	0.43
" 29	455	2.74
" 30	690	3.70
July 1	777	1.80
" 2	627	4.32

The glycosuria seemed likely to persist on the lung diet, but promptly ceased with a single day of fasting on July 3. Fasting was continued on July 4 and 5 to restore the damaged tolerance. Glycosuria was then absent on 1 kg. of beef lung daily till July 9, when there was a sudden appearance of 2.17 per cent. of sugar in 241 cc. of urine. Another 3-day fast was then imposed to stop glycosuria and improve tolerance, and the dog was subsequently used for other experiments.

A possible interpretation of these results seems to be that the glycosuria of June 4 represented a cumulative effect of the preceding thyroid diet; that glycosuria was then absent on the usual kilogram of lung, but was produced within two days by the change to a kilogram of thyroid. As usual when the tolerance is injured, this glycosuria continued after the cause was removed, so that fasting was necessary and the tendency to glycosuria continued for some time on the lung diet formerly tolerated. There were no other perceptible symptoms from the thyroid feeding.

Cat B2-01.—A large strong black-and-white female in excellent condition at a weight of 4.1 kg., was partially depancreatized on Feb. 19, 1914. The tissue removed weighed 7.2 gm., and the remnant about the main duct was estimated at 1.8 gm. (1/5). Glycosuria followed, but was checked by fasting and undernutrition. At first the protein tolerance was limited and heavy glycosuria resulted from any considerable quantities of meat, but by April the animal had become able to live on beef *ad libitum* without excretion of sugar though milk still caused glycosuria. The removal of 0.25 gm. of additional pancreatic tissue on April 21 failed to halt the rise of tolerance, and by the middle of May the cat was able to take 300 cc. milk and as much meat as she would eat daily without glycosuria. This gain of tolerance, though doubtless due chiefly to pancreatic regeneration (the remnant being finally found to weigh 2.6 gm. at autopsy), may have been connected partly with the fall in weight, which now remained almost constant at 3.5 kg.

May 21, the addition of 50 gm. glucose to the usual milk brought back heavy glycosuria. The tolerance was broken down by this program, so that after June 12 the glucose could be omitted and heavy glycosuria continued on a diet of beef and milk. The animal was then kept in a border-line state and used for several experiments, showing the effect of alterations of body weight on the food tolerance. At a weight of 3.3 kg., glycosuria was absent on a diet of 300 gm. lean meat and 300 cc. milk, but it was present even without milk when the weight rose to 4.5 kg.

March 20, 1915, the weight was 2.8 kg., and a diet of 200 gm. beef lung was begun. March 30, this was changed to 100 gm. lung and 100 gm. fresh sheep thyroid, and on April 2 to 200 gm. thyroid. Glycosuria remained absent, and the fasting blood sugar on April 6 was 0.095 per cent. The weight had risen to 3.2 kg.

April 9 to 23, the diet was 300 gm. beef lung without thyroid, and the weight rose to 3.5 kg., with no glycosuria. April 23, this diet was changed to 100 gm. lung and 200 gm. fresh sheep thyroid, and on April 26 to 300 gm. thyroid. There was no glycosuria, and the weight remained practically constant. The same condition persisted with a change to 300 gm. lung, May 17–23, and a return to 300 gm. thyroid, May 24–25.

May 26, feeding of 400 gm. thyroid daily was begun, and slight glycosuria appeared May 28. The further results are shown in Table I.

The glycosuria continued, and by June 24 had increased to 2.22 per cent. in 240 cc. urine, the body weight being 4.4 kg. Milk was then omitted, but equally heavy glycosuria persisted on the diet of 400 gm. beef lung. By July 1 the glycosuria was 2.86 per cent. in

TABLE I.

Cat B2-01.

Date.	Weight.	Diet.	Urine.	
			Vol.	Sugar.
1915	*kg.*		*cc.*	*per cent.*
May 28	3.5	400 gm. sheep thyroid	225	0.28
" 29		" " " "	210	0.30
" 30		" " " "	235	0.62
" 31		" " " "	405	0.50
June 1	3.6	" " " "	250	0.43
" 2		" " " "	170	0.33
" 3		" " " "	182	0.40
" 4	3.6	" " " "	196	0.18
" 5		400 gm. beef lung	435	0
" 6		" " " "	265	0
" 7		" " " " and 25 cc. milk	425	0
" 8		" " " " " 50 " "	180	0
" 9		" " " " " 100 " "	215	0
" 10	3.8	" " " " " 150 " "	175	0
" 11		" " " " " 200 " "	186	0
" 12		" " " " " 250 " "	210	0
" 13		" " " " " " " "	290	0.52
" 14	4.2	" " " " " " " "	240	0.52
" 15		" " " " " " " "	285	0.77

205 cc. urine, and the weight was 4.3 kg. Continuous fasting from July 1 to 18 was then necessary to stop the glycosuria. This reduced the weight to 3.1 kg. Thereafter a diet was gradually built up, and the cat finally became able again to tolerate 300 to 400 gm. lung daily at about 3 kg. weight.

In this experiment large quantities of thyroid were necessary for any effect, but this was definite when obtained. The glycosuria

present on feeding 400 gm. of thyroid was absent on 400 gm. of lung, even with addition of as much as 200 cc. of milk. The thyroid glycosuria remained slight, however, while that resulting from the increase of milk to 250 cc. soon became heavy, and was also harder to stop by fasting. No other symptoms of hyperthyroidism were perceptible, and the body weight increased with increase of the quantity of thyroid fed.

Cat B2-12.—Adult male, black-and-white, weight 3.8 kg. Nov. 5, 1914, removal of pancreatic tissue weighing 9.7 gm. Remnant left about main duct estimated at 2.6 gm. (a little over 1/5). Feeding tests up to March, 1915 demonstrated that glycosuria was absent on the largest quantities of meat which the cat would eat, but heavy glycosuria could regularly be produced by addition of milk. In March the feeding of meat *ad libitum* was replaced by a fixed diet of 400 gm. beef lung. Table II, gives the record after introduction of fresh sheep thyroid. No blood sugar data were obtained, except a normal figure of 0.10 per cent. fasting on April 6.

This cat failed to show glycosuria at the normal weight of 3.8 kg. or less, on either lung or thyroid feeding. Fattening to a maximum weight of 4.5 kg., by addition of such quantities of lard as the animal would eat, also led to no glycosuria with either lung or thyroid. Beef heart, however, for some reason gave rise to definite glycosuria in the obese animal, but failed to do so after the tolerance was raised by reduction of body weight. As these tests show how close the animal was to the verge of glycosuria, the absence of glycosuria with thyroid feeding represents a very decided negative result in this experiment. There were also no other perceptible symptoms of thyroid excess.

Dog D4-77.—A small thin black collie mongrel weighing 11 kg., was partially depancreatized on Jan. 19, 1917. The tissue removed weighed 34.6 gm., and the remnant left about the main duct was estimated at 4.3 gm. (1/9). Heavy glycosuria ensued on diets of 500 gm. beef lung or less. By fasting and reduced diet the weight was reduced as low as 7.5 kg. in March. The limits of tolerance were definitely established by repeated tests, and the nutrition was grad-

ually built up by protein-fat diets. Glycosuria always resulted from feeding 500 gm. of lung, but 300 gm. lung and 100 gm. suet appeared to be well tolerated.

TABLE II.

Cat B2-12.

Date.	Weight.	Diet.	Urine.	
			Vol.	Sugar.
1915	*kg.*		*cc.*	*per cent*
Mar. 30	3.4	300 gm. lung and 100 gm. thyroid.	210	0.60
" 31		" " " " " " "	205	0.28
April 1	3.6	" " " " " " "	110	1.88
" 2		" " " " " " "	200	1.40
" 3		" " " " " " "	156	0.27
" 4		" " " " " " "	170	1.02
" 5		" " " " " " "	237	0.18
" 6	3.8	" " " " " " "	Lost	—
" 7		400 gm. lung.	280	0
" 8 to 23		" " "	—	0
" 24 to 26	3.8	200 gm. lung and 200 gm. thyroid.	—	0
" 27 to 29		400 gm. thyroid.	—	0
" 30 to May 7	3.7	400 gm. lung.	—	0
May 8 to 17	3.7	400 gm. thyroid.	—	0
" 18 to 24	3.7	500 gm. lung.	—	0
" 25 to 31	3.7	500 gm. lung and lard (unweighed).	—	0
Jun. 1 to 7	3.8	500 gm, beef heart and lard.	—	0
" 8 to 25	4.5	" " " " " "	—	0
" 26	4.5	" " " " " "	223	0.55
" 27	4.5	500 gm. lung and lard.	162	1.22
" 28	4.5	500 gm. beef heart and lard.	141	0.50
" 29 to Jul. 2	4.5	500 gm. lung and lard.	—	0
Jul. 3	4.5	" " " " "	230	1.42
" 4	4.5	500 gm. thyroid, gradually reduced to 250 gm.	197	0.61
" 5 to 14	4.5	because of growing dislike for thyroid.	—	0
" 15 to 27	3.1		—	0
" 28 to Aug. 4	3.0	500 gm. beef heart.	—	0

Dog D4-89.—A mongrel was picked for close resemblance in size and form to serve as a normal control, but was fatter than dog D4-77 so that the initial weight was 15 kg. The opportunity was taken to perform some phlorizin experiments upon D4-89, which subjected

this dog to undernutrition and glycosuria somewhat similar to those of D4-77.

April 6 the two dogs, free from glycosuria and weighing respectively 9.2 and 10.7 kg., were placed on a diet of 300 gm. beef lung and 100 gm. suet, with a trifle of talcum powder to prevent diarrhea. Beginning April 10, the urinary nitrogen was determined by Kjeldahl for a preliminary period of 2 days, and then thyroid tablets were added to the diet, as shown in Table III. The ordinary commercial 2-grain tablets were used, and were eaten eagerly by both dogs. Glycosuria occurred in dog D4-77 on April 13 and 14, and belated attention was then given to the composition of the tablets, which were found to give a heavy copper reduction, evidently due to milk sugar. The glycosuria ceased with the feeding of 100 gm. suet and 50 thyroid tablets (without lung) on April 14 and 15, and only 100 gm. suet the next day.

A fresh start was made on April 17, with the regular diet of 300 gm. lung and 100 gm. suet, together with 10 gm. of Armour's desiccated thyroid powder, which was labelled as containing 0.2 per cent iodine and was found free from starch or sugar by test. The experimental results may be summarized under 3 headings.

Diabetic Symptoms.—The thyroid dosage produced both hyperglycemia and glycosuria in dog D4-77, but neither in the normal control D4-89. All blood samples were taken before the day's food was given. The plasma was always clear in dog D4-89, but sometimes showed more or less abnormal turbidity from fat in dog D4-77. There was, however, no apparent relation between this lipemia and the thyroid feeding, and it was probably related only to the diabetic state.

Other Symptoms of Thyroid Excess.—No distinct effect of any kind was noticeable in dog D4-89. The animal was not nervous but may have been slightly unwell. The temperature, taken by rectum several times daily, was regularly between 38° and 38.6°C., while the pulse taken with the animal as quiet as possible varied between 100 and 136. Only once was the temperature found as high as 39° and the pulse 152. There was no consistent increase of urinary nitrogen under thyroid administration. Neither animal was catheterized, and the feces were not analyzed; but the feces were uniformly well formed and apparently well digested, and the record of cage urine indicates

TABLE III.

Date.	Dog D4-77. Weight.	Blood. Plasma sugar.	Blood. Lipemia qual.	Urine. Vol.	Urine. Dext.	Urine. T. N.	Dog D4-89. Weight.	Blood. Plasma sugar.	Blood. Lipemia qual.	Urine. Vol.	Urine. Dext.	Urine. T. N.	Diet.
1917	*kg.*	*per cent.*		*cc.*	*gm.*	*gm.*	*kg.*	*per cent.*		*cc.*	*gm.*	*gm.*	
Apr. 10–11	9.3	—	—	768	0	6.38	10.9	—	—	480	0	5.64	300 gm. lung and 100 gm. suet.
" 11–12	9.5	0.124	0	388	0	4.06	10.8	0.086	0	665	0	7.20	Same.
" 12–13	9.4	—	—	250	0	4.58	10.9	—	—	624	0	7.18	" plus 30 two-grain thyroid tablets.
" 13–14	9.3	—	—	540	13.80	7.38	10.8	—	—	771	0	6.59	" plus 50 tablets.
" 14–15	9.3	—	—		5.10	4.10	10.5	—	—	770	0	4.34	100 gm. suet and 75 thyroid tablets.
" 15–16	9.2	—	—	386	0	2.38	10.3	—	—	540	0	2.04	100 gm. suet and 50 thyroid tablets.
" 16–17		0.204	0	1138	0	3.64		0.116	0	712	0	4.05	100 gm. suet only.
" 17–18	8.9	0.118	Heavy.	1220	0	5.40	10.2	—	—	778	0	6.94	300 gm. lung, 100 gm. suet and 10 gm. thyroid powder.
" 18–19		—	—	1720	0	7.40		0.070	0	962	0	8.86	Same.
" 19–20	9.0	0.175	Slight.	1916	0	7.20	10.4	0.074	0	577	0	3.85	"
" 20–21		0.208	Faint.	1910	6.40	6.32		0.081	0	456	0	5.70	"
" 21–22	9.2	—	—	1410	7.50	9.06	10.2	—	—	340	0	4.93	"
" 22–23		—	—	1390	+	7.56		—	—	176	0	2.47	"
" 23–24	9.0	—	—	935	8.60	6.04	10.5	—	—	370	0	5.34	"
" 24–25	9.2	—	—	825	6.19	6.40	10.5	—	—	315	0	5.52	"

fairly regular voiding. The diabetic dog D4-77 was by nature a more nervous animal. A nervous influence of the thyroid dosage was unmistakable, in the form of extraordinary excitability, restlessness, and sometimes tremors. The rectal temperature during the experimental period was between 39° and 39.6°C. The pulse was generally about 164, only once as low as 148. The urinary nitrogen was distinctly increased during the experimental period, but some allowance must be made for the nitrogen of the 10 gm. of thyroid powder. Neither dog showed exophthalmos, and neither lost weight during the experimental period more than could be accounted for by the reduced diet of April 14 to 16.

After-Period.—An after-period would have been desirable in the table, to show the effect of simple omission of thyroid upon the sugar and nitrogen excretion, with the diet and other conditions unchanged. It was known from previous experience, however, that a glycosuria which had gained such headway would not stop with simple withdrawal of thyroid on a diet so near the border of tolerance, but on the contrary would quickly progress into hopeless diabetes. Therefore thyroid was discontinued, and 2 fast-days and a day of only 100 gm. suet were used to stop the glycosuria in dog D4-77. The diet of 300 gm. lung and 100 gm. suet was then once more tolerated. Meantime the nervous symptoms subsided and the pulse and temperature returned to the same normal level as in dog D4-89.

It is not certain whether the greater nervous response to thyroid on the part of the diabetic dog in this experiment was due entirely to the more nervous constitution or partly to a sensitive state created by diabetes. The essential feature of the results was the production of hyperglycemia and glycosuria by thyroid in a potentially diabetic dog and the absence of these in a normal dog.

Dog E5-19.—A thin brindle female mongrel weighing 10 kg., was partially depancreatized on Sept. 28, 1917. The tissue removed weighed 28.3. The remnant about the main duct was estimated at 2.4 gm. (about 1/13). The resulting diabetes was severe and required stringent undernutrition for its control. When the body weight had been reduced to 9 kg., 200 gm. of lung still caused glycosuria, but a diet of 100 gm. lung and 100 gm. suet was tolerated.

TABLE IV.

Dog E5-19.

Date.	Time.	Plasma.			Urine.					Remarks.
		Sugar mg. per 100 cc.	CO_2 vol.	Corp. vol.	Vol.	Sugar.	Acetone qual.	Total—N	NH_3—N	
1917			*per cent.*	*per cent.*	*cc.*	*gm.*		*gm.*	*gm.*	
Nov. 3	—	—	—	—	110	Negative.	Negative.	2.94	0.24	Fed 150 gm. lung, 100 gm. suet.
" 4	—	—	—	—	111	"	"	3.44	0.18	" 150 " " 100 " "
" 5	—	—	—	—	120	"	"	4.30	0.28	" 150 " " 100 " "
" 6	—	—	—	—	100	"	"	3.54	0.29	" 150 " " 100 " "
" 7	—	—	—	—	100	"	"	4.02	0.22	" 150 " " 100 " "
" 9	—	—	—	—	100	"	"	3.56	0.19	" 150 " " 100 " "
" 10	—	—	—	—	130	"	"	4.97	0.26	" 150 " " 100 " "
" 11	—	—	—	—	65	"	"	2.97	0.16	" 150 " " 100 " "
" 12	—	—	—	—	60	"	"	2.66	0.15	" 150 " " 100 " "
" 13	10:15 a.m.	179	53.6	35.6	75	"	"	2.53	0.21	" 150 " " 100 " "
	2:15 p.m.	172	59.4	37.5	—			—	—	
	6:15 p.m.	116	59.5	31.2	—			—	—	
" 14	—	—	—	—	250	"	"	4.44	0.66	Same plus 5 gm. thyroid powder.
" 15	—	—	—	—	130	"	"	2.66	0.66	" " 5 " " "
" 16	—	—	—	—	200	"	"	5.12	0.58	" " 5 " " "
" 17	—	—	—	—	240	"	"	9.69	0.75	" " 5 " " "
" 18	—	—	—	—	60	"	"	2.11	0.39	" " 5 " " "
" 19	—	—	—	—	330	"	"	6.76	0.56	" " 5 " " "
" 20	—	—	—	—	400	"	"	5.36	1.12	" " 5 " " "
" 21	10:00 a.m.	196	67.1	34.5	400	"	"	8.48	0.68	" " 5 " " "
	4:00 p.m.	250	50.0	34.5	—					
	6:00 p.m.	250	64.2	34.0						
" 22	—	—	—	—	570	"	"	6.96	—	" " 10 " " "
" 23	—	—	—	—	720	"	"	6.40	0.61	" " 10 " " "
" 24	—	—	—	—	630	"	"	5.53	0.98	" " 10 " " "
" 25	—	—	—	—	600	"	"	7.62	1.02	" " 10 " " "
" 26	—	—	—	—	1000	"	"	12.28	1.40	" " 10 " " "
" 27	—	71	51.6	15.4	670	"	"	8.54	0.53	Autopsy blood.

Accordingly this was instituted as a regular diet on Oct. 31. The results of the subsequent experimental period are shown in Table IV.

After the preliminary period from Nov. 3 to 13, Armour's thyroid powder, of 0.2 per cent. iodine content, was added to the diet, first in 5 gm. and later in 10 gm. quantity. The dog developed diarrhea, emaciated from an initial weight of 9 kg. to a final weight of 6.5 kg., and died of inanition on Nov. 27.

Glycosuria was not produced, but the higher blood sugar levels during digestion of the regular diet on Nov. 21 as compared with Nov. 13 seem to indicate a definite influence of the thyroid, especially in view of the indigestion and emaciation.

The thyroid feeding failed to cause evident nervousness in behavior, probably because of the weakness. The rectal temperature, instead of being subnormal as usual in cachectic states, was between 38.6 and 39°C. during the period of thyroid dosage. The pulse was generally 164. The dog's skin was noticeably hot to touch.

The urine was passed spontaneously. The elevations of ammonia on Nov. 20, 25 and 26 may have been due to fermentation, which on the whole was successfully guarded against by the use of toluene and sulphuric acid in the bottles. The excretion of urinary nitrogen was considerably higher after thyroid administration than before. The high figures for Nov. 26 and for the partial specimen on Nov. 27 evidently represent a premortal rise.

It will be noticed that no acidosis was produced in this or other experiments either by the thyroid or by the high fat content of the diet.

At autopsy, the thyroid was found to consist of large vesicles distended with colloid, with their margins thickly beaded with vacuoles (active secretion?). The liver, adrenals and spleen were normal. The kidneys were normal with the possible exception of slight vacuolation of Armanni character in certain tubules. The pancreas remnant was fully normal, and in particular was free from vacuolation or other changes in the islands. Thyroid intoxication thus showed no specific effect upon the pancreatic structure.

2. *Experiments with Thyroidectomy.*

Dog B2-89.—A yellow female mongrel, aged 4 years, with an initial weight of 13.2 kg., had been kept under observation in a condition bordering on diabetes and used for various experiments since April 12, 1915. Actual diabetes was produced by the removal of 0.25 gm. additional pancreatic tissue on March 16, 1916. May 18, 1916, both ovaries were removed, as previously described.[55] The diabetes was mild, so that indefinite quantities of protein could be tolerated, as also approximately 150 gm. of bread, but the addition of 75 gm. glucose regularly caused glycosuria of 12 or 13 gm. The oöphorectomy produced no lasting change in this tolerance.

June 20, 1916, the entire thyroid was removed, with the exception of such tiny shreds as had to be left in order to save three parathyroids with their circulation. The dog quickly recovered from the ether and ate the regular diet on the operative day and the following days. Glycosuria resulted, however, first in traces of 0.2 to 0.3 per cent., but increasing by June 29 to 2.70 per cent. in 380 cc. urine. Therefore, after glycosuria was stopped by a fast-day on June 30, the diet was changed to 100 gm. lung, 100 gm. suet and 50 gm. bread, on which glycosuria remained absent. After July 6 the diet with 150 gm. of bread was again tolerated without glycosuria.

July 27, 75 gm. of glucose was added to the diet, with resulting excretion of 9.1 gm. in 217 cc. urine. July 28, the same test was repeated, with excretion of 8.15 gm. in 392 cc. urine.

At the time of the thyroidectomy on June 20, the dog was thin at a weight of 8.3 kg., because of reduced diet, but was strong and lively. After the operation there was increasing indigestion and bulky feces, with corresponding emaciation. At the time of the glucose tests mentioned, the weight was down to 7.0 kg., and the slightly lower glycosuria as compared with former tests is amply explained by the lower weight and poorer food absorption. Glycosuria was absent on the regular diet thereafter, until death from inanition on Aug. 4, at a weight of 6.8 kg.

At autopsy, one small nodule was found at the site of each thyroid lobe, not much larger than a normal parathyroid. These were not examined microscopically, but it was evident that the thyroid had not

regenerated to any important extent. With the exception of moderate fatty infiltration of liver and kidneys, the viscera were negative. The pancreas remnant was small, nodular and hard, but when it was cut open the interior was found to be composed of softer tissue. Microscopic examination confirmed the existence of a patchy fibrosis, with interspersed areas of normal appearing acinar tissue. Islands of Langerhans were fairly numerous but small, as frequently found in such a remnant. Their cells were normal and free from vacuolation or any other changes attributable to the thyroidectomy.

The essential feature of this experiment was that the glucose tolerance, though lowered temporarily by the operation (possibly by trauma to the parathyroids), was not increased by thyroidectomy.

Dog D4-62.—This dog, possessing one-tenth of the pancreas, was also previously described in connection with an oöphorectomy which failed to alter the food assimilation.[55] Protein food could be tolerated in maximum quantities, up to 1 kg. of beef lung daily, but the addition of 50 gm. bread sufficed to cause heavy glycosuria. Feb. 3, 1917, the left lobe of the thyroid was removed, leaving the one prominent parathyroid *in situ*. The usual diet of 1 kg. of lung was eaten on this and the following days without glycosuria. Also the addition of 50 gm. of bread on Feb. 7, 8 and 9 resulted in no glycosuria, but on Nov. 10 it gave rise to 1.69 per cent. of sugar in 456 cc. of urine. Similar glycosuria now persisted on the diet of 1 kg. of lung without bread, and twice returned after being stopped by fast-days, so that on Feb. 20 the diet had to be changed to 500 gm. lung and 100 gm. suet, on which glycosuria remained absent. These fluctuations of tolerance are within the limits of ordinary variation in such animals. The apparent rise is explained by a preceding period of aglycosuria, and the subsequent fall is accounted for by the preceding period of 10 days in which glycosuria had been present most of the time.

To control possible differences in absorption, a comparison was made of intravenous glucose tests on Jan. 31, before the partial thyroidectomy, and on Feb. 28. The weight on the former date was 13.5 kg. and on the latter date 12.5 kg., but the dosage was 1 gm. per kg. based on the original normal weight of 19.1 kg. The amount of 19.1 gm. of Merck anhydrous glucose was weighed out and dis-

solved in water to make a 20 per cent. solution. This quantity was given each hour by the method of interrupted injections used in several of these series of experiments. The dog was catheterized, blood was drawn from a previously exposed jugular vein, and by an exchange of syringes the sugar injection was given through the same needle. Every 20 minutes thereafter the same program was repeated, one-third of the hourly dose of glucose being injected each time. The results are shown in Table V.

TABLE V.

Dog D4-62.

Jan. 31, 1917.			Feb. 28, 1917.			Time.
Plasma sugar.	Urine.		Plasma Sugar.	Urine.		
	Vol.	Glucose.		Vol.	Glucose.	
per cent.	*cc.*	*per cent.*	*per cent.*	*cc.*	*per cent.*	
0.100	124	0	0.088	158	0	Before 1st injection.
0.196	10	2.94	0.210	20	4.6	" 2nd "
0.385	21	3.23	0.345	31	4.75	" 3rd "
0.385	25	4.35	0.357	34	4.03	" 4th "
	21	5.56	0.384	36	4.14	" 5th "
0.455	28	6.25	0.400	40	4.03	" 6th "
0.455	27	5.41	0.486	38	3.86	" 7th "
						Injections discontinued.
0.416	33	4.54	0.384	49	6.17	20 min. after 7th injection.
0.286	21	3.57	0.212	32	4.60	40 " " " "
0.384	4	2.08	0.185	7	3.47	60 " " " "
0.137	7	Trace.	0.170	Not catheterized.		80 " " " "
0.088	10	0	0.149	36	2.08	100 " " " "

The tests showed no difference of glucose assimilation before and after removal of one lobe of the thyroid. As the suggestions of clinicians for the modification of human diabetes have pertained to partial, not total thyroidectomy, this test of the influence of partial thyroidectomy was undertaken with this point in view.

Dog D4-73.—A brindle female mongrel aged 6 years, weighing 17.25 kg., was partially depancreatized on Jan. 17, 1917. The tissue removed weighed 36.4 gm., and the remnant about the main duct

was estimated at 4.8 gm. (1/8-1/9). Glycosuria could at first be produced only by addition of glucose to the diet. After the tolerance was thus reduced, the dog was used for prolonged high fat diets, sometimes with and sometimes without glycosuria, in an unsuccessful attempt to produce acidosis, as will be described elsewhere. At first the body weight was thus raised above 20 kg. Later it fell with progress into severe diabetes. In the latter part of July and early days of August it approximated 15 kg. The dog had reached a dangerously cachectic state, so that no prolonged fasting could be endured, and it was improbable that even the longest fasting could halt the glycosuria. Therefore on Aug. 10 complete thyroidectomy was performed, in order to learn whether any practical benefit could thus be attained in the direction of saving the dog's life. The results are shown in Table VI.

One large parathyroid was left on each side with its blood supply intact. Both thyroid lobes were dissected out completely with the exception of tiny shreds adhering to the parathyroids or their vessels. No symptoms of tetany or other disturbances followed the operation. The diet consisted chiefly of suet both before and after the operation in order to supply a maximum of nutrition in the form of fat together with a minimum of sugar-forming material. It was eaten and digested well up to Aug. 14. The dog then became too weak to eat, and died in the usual cachexia on Aug. 16. The thyroidectomy may have hastened death by a day or two, but had no other evident effect. The decline of glycosuria and hyperglycemia was no greater than is known to occur in the terminal cachexia of many severely diabetic animals.

The gross autopsy was normal. The two parathyroids appeared normal, and microscopically were found accompanied by a few large thyroid vesicles, full of colloid. The pancreas remnant, normal in appearance and consistency, weighed 5.2 gm. Microscopically it showed very slight fibrosis, not involving the islands. The islands were on the point of disappearing through hydropic degeneration, being scarce, small, and composed of maximally vacuolated cells. The practical absence of functional island tissue made evident the futility of attempting to aid such an animal by thyroidectomy.

TABLE VI.

Dog D4-73.

Date.	Weight.	Urine.					Blood.			Remarks.
		Vol.	Sugar.	Acetone qual.	Total —N	NH_3 —N	Plasma sugar mg. per 100 cc.	Plasma CO_2 vol.	Corp. vol.	
1917	*kg.*	*cc.*	*gm.*		*gm.*	*gm.*		*per cent.*	*per cent.*	
Aug. 4	—	806	8.60	Slight.	3.84	0.44	—	—	—	Fed 100 gm. suet.
" 5	—	1164	9.24	Moderate.	3.60	0.53	—	—	—	" 100 " "
" 6	—	1070	Very faint.	Faint.	2.16	0.43	—	—	—	Not fed.
" 7	—	910	Negative.	Negative.	2.48	0.24	244	63.3	44.3	Fed 100 gm. suet.
" 8	15.8	888	"	"	2.44	0.28	145	68.1	37.5	" 200 gm. lung and 100 gm. suet.
" 9	—	1095	Doubtful.	Slight.	2.40	0.38	263	—	43.1	Not fed.
" 10	15.6	1125	Faint.	Faint.	3.60	0.29	151	47.1	48.0	" "
" 11	—	380	Negative.	Negative.	0.14	0.95	278	51.0	39.8	" "
" 12	—	480	"	"	0.28	3.85	—	—	—	Fed 100 gm. lung and 100 gm. suet Vomited diet.
" 13		795	Faint.	Faint.	0.32	3.60	333	—		Not fed.
" 14	—	790	"	"	2.56	0.26	384	—	—	Fed 50 gm. suet. Vomited.
" 15	—	558	"	Negative.	3.66	1.80	—	—	—	Not fed.
" 16	—	435	Very faint.	Faint.	3.75	1.70	357	50.0	—	" "

Dog E5-97.—A brown male bulldog mongrel aged 6 years and weighing 17.5 kg., was partially depancreatized on Sept. 28, 1917. The tissue removed weighed 44.0 gm., and the remnant about the main duct was estimated at 4.7 gm. (1/10–1/11). Tests then showed the susceptibility to prompt and heavy glycosuria on either bread or meat feeding, but the diabetes was kept under control by fasting and very low protein-fat diets.

Nov. 1, when the body weight had been reduced to 13.5 kg., an intravenous glucose tolerance test was performed. Feeding tests during the following days showed that the undernutrition had raised the dog's tolerance to such a point that as much as 1 kg. of beef lung and 100 gm. of suet could be eaten without glycosuria. A standard diet of 600 gm. lung and 100 gm. suet caused no hyperglycemia when taken for a week. The addition of 50 gm. bread for 1 day caused no glycosuria, but an increase to 100 gm. of bread resulted in slight glycosuria the first day and heavy glycosuria (2.9 per cent. in 1040 cc. urine) the second day.

Nov. 21, with normal urine and blood on the standard diet, and with body weight of 13.7 kg., the thyroid was removed as completely as possible without damaging one large parathyroid which was left on each side. Slight glycosuria occurred during a few hours after operation, but on the following days the standard diet was taken without apparent abnormalities of any kind.

Nov. 28, the standard diet of 600 gm. lung and 100 gm. suet was increased by 50 gm. bread. Nov. 29, the bread was increased to 100 gm., still without glycosuria. Nov. 30, an increase to 150 gm. bread caused an excretion of 2.7 per cent. glucose in 350 cc. urine. Glycosuria then remained absent on the standard diet. Beginning Dec. 8, 100 gm. bread was again added daily, with the result of slight glycosuria for 2 days and absence of glycosuria for the ensuing 3 days. Dec. 13, a little milk was given in addition to the lung-suet-bread diet, still without glycosuria. The standard lung-suet diet was then resumed.

Dec. 20, when the body weight was 13.6 kg. and the general health apparently good, another intravenous tolerance test was performed. The same test was repeated on April 21, when the weight was 13.0 kg. Blood sugar analyses on other days will be mentioned below.

Jan. 15, 1918, the body weight was 13.0 kg., and the daily addition of 5 gm. of thyroid powder (Armour's) to the standard diet was commenced. By Jan. 28 the body weight had fallen to 12.25 kg. and the dog seemed distinctly weaker. The rectal temperature was not above 38.8°C. The heart rate at rest was ordinarily only 120 to 130, but the rhythm was more irregular than observed in normal dogs, and slight exertion caused undue acceleration of the rate. Beginning Feb. 5, the standard diet was continued without thyroid. By Feb. 20 the body weight had risen to 12.9 kg. and the general condition appeared better.

Beginning Feb. 21, the diet was increased to 1200 gm. lung and 100 gm. suet, except on days when the standard diet was resumed for blood sugar analyses. The feces had gradually been growing more bulky for some time past, and though the dog ate the increased diet eagerly, digestion and absorption continued to fail, so that by April 17 the weight was still only 13 kg. From April 18 to 21 inclusive, the diet of 1200 gm. lung and 100 gm. suet was increased by the addition of 100 gm. bread. Glycosuria was absent until the last day, when there was 0.95 per cent. glucose in 550 cc. urine. Bread was then omitted and the glycosuria ceased.

The feces continued to grow more bulky and foul, until they obviously contained most of the protein and fat of the diet. By May 18, the weight had fallen to 9.3 kg., and the dog was moribund from weakness. Intelligence and courage were preserved; there was no physical or psychic change suggestive of myxedema. The penis hung protruded from the prepuce, and the exposed portion of it was black with dry gangrene. The rectal temperature was below 33°C. The pulse was 69 per minute and very feeble. The respiration was only 8 per minute, but was dyspneic. Inspiration was prolonged until the chest was distended to its utmost capacity; expiration was protracted and forcible, squeezing out all possible air by a maximum effort of contraction. There were no pauses between respiratory movements. The urine remained free from sugar and acetone. The blood findings were as follows: corpuscle volume, 35.5 per cent.; plasma sugar 0.27 per cent.; plasma acetone negative (nitroprusside qualitative and Van Slyke quantitative); CO_2 capacity of plasma 32.8 volume per cent. All these figures, obtained in the forenoon, were practically duplicated

in the blood taken at autopsy in the evening. The reason for the high level of plasma sugar is unknown. The low bicarbonate concentration, with other tests for acidosis negative, is perhaps explained by dyspnea according to the work of Yandell Henderson.

The autopsy obtained at 9 p.m. was grossly negative except for extreme emaciation. The remains of thyroid-parathyroid tissue obtained on the two sides weighed 0.2 gm. in total. The pancreas remnant, normal in appearance and consistency, weighed 3.7 gm. The reduction as compared with the 4.7 gm. estimated at the original operation may be sufficiently explained by the wasting of all organs in cachexia. Microscopically normal parathyroids were found, with only a trifle of thyroid tissue adjoining them. This latter tissue seemed to consist of interlacing cords of cells, with only occasional small vesicles containing a trifle of colloid. The pancreatic tissue was normal except for a slightly foamy appearance of the cytoplasm of both island and acinar cells, probably connected with the cachexia. The islands otherwise were normal in number, architecture and cytology, and hydropic degeneration was absent. The other viscera showed no more than simple atrophic changes.

The influence of thyroidectomy upon the carbohydrate assimilation was judged by two criteria, namely by intravenous glucose tests and by the hyperglycemia following a protein-fat diet. The glucose tests are shown in Table VII.

The intravenous tolerance tests were used to exclude irregularities of intestinal absorption. The method employed was that of discontinuous injections, as described in several previous papers. An external jugular vein of the fasting animal was painlessly exposed about an hour before the beginning of the experiment. A solution containing 20 per cent. by weight of Merck's anhydrous glucose was prepared. The dosage was arbitrarily chosen as 1 gm. per kg. per hour on the basis of 14 kg. body weight. In beginning the experiment, the dog was catheterized, a blood sample was drawn from the exposed jugular with a syringe, and an injection of one-fourth of the hourly dose (3.5 gm. glucose, or 17.5 cc. of solution) was given with a different syringe through the same needle. Further injections were given at the end of 15, 30 and 45 minutes; but instead of taking blood and urine samples each 15 minutes, as usual, these were taken only

TABLE VII.

Dog E5-97. Intravenous Glucose Tests.

Time.	Plasma sugar, mg. per 100 cc.			Urine.						Remarks.
				Nov. 1, 1917.		Dec. 20, 1917.		April 21, 1918.		
	Nov. 1, 1917.	Dec. 20, 1917.	April 21, 1918.	Vol.	Sugar.	Vol.	Sugar.	Vol.	Sugar.	
p.m.				*cc.*	*gm.*	*cc.*	*gm.*	*cc.*	*gm.*	
4:05	95	172	146	70	Negative.	78	Negative.	30	Negative.	Blood drawn before injection.
4:25										Given 1st injection.
4:40										" 2nd "
4:55										" 3rd "
5:10										" 4th "
5:20	286	455	370	No urine.		92	0.38	90	3.47	
5:25										" 5th "
5:40										" 6th "
5:55										" 7th "
6:10										" 8th "
6:25	294	370	400	70	1.71	22	0.87	60	2.73	15 min. after last injection.
7:25	164	286	228	No urine.		No urine.		40	1.25	80 " " " "
8:25	145	149	137	40	0.59	17	Negative.	32	0.27	140 " " " "

hourly, for the two hours during which injections were given, and for the following two hours.

Three identical tests were performed, namely on Nov. 1 before thyroidectomy, on Dec. 20 early in the period after thyroidectomy, and on April 21 late in the period after thyroidectomy. The two latter tests indicated a distinct fall of tolerance as compared with the first test, judged especially by the curve of hyperglycemia. The excretion of glucose was slightly less on Dec. 20 than on Nov. 1, but on April 21 was much higher. The apparent lowering of assimilation may have been due to cachexia or any other accidental cause, but it must at least be concluded that the tolerance according to the intravenous tests was not raised by thyroidectomy.

The second criterion of assimilation adopted was the influence of a standard mixed meal, consisting of 600 gm. lung, 100 gm. suet, and

TABLE VIII.

Dog E5-97. Feeding Tests.

Plasma sugar, mg. per 100 cc.

	Nov. 14, 1917.	Dec. 27, 1917.	Jan. 15, 1918.	Jan. 28, 1918.	Feb. 4, 1918.	Feb. 11. 1918.	Mar. 7, 1918.	Apr. 17, 1918.
Before feeding.....	126	151	133	143	167	152	128	125
3 hrs. after feeding.....	183	161	119	103	149	182	132	116
6 " " "	174	161	149	161	161	—	115	119

50 gm. bread. On selected days this was substituted for the regular diet, and analyses of blood and urine were obtained before feeding and 3 and 6 hours after. No glycosuria occurred on any occasion, but on the whole the plasma sugar curve was lower in the various tests after thyroidectomy than it had been on Nov. 14, before thyroidectomy. It will be seen in Table VIII that the plasma sugar held its lowest values on March 7 and April 17, after cachexia had become marked. The results of the intravenous tests indicated that the dog's ability to take higher diets of carbohydrate and protein with less tendency to hyperglycemia or glycosuria was not due to a true increase of tolerance. On the other hand the character of the feces and the stationary or falling weight with increased diets served to explain the lower sugar curves after feeding as due to impaired absorption. This finding is not contrary to the observation of Janney and Isaacson that absorption

is not impaired with thyroid deficiency, for in the present instance thyroid and pancreatic deficiency coexisted and the results suggested that the combination was more serious in all respects than the deficiency of only one organ.

The feeding of 5 gm. powdered thyroid daily from Jan. 15 to Feb. 4 did not restore any better health. There were some indications that this quantity represented a toxic excess of thyroid, but the important feature is that such a quantity failed to cause glycosuria at any time or any special hyperglycemia in the feeding test of Feb. 4 (Table VIII), though the dog was actually diabetic. This observation therefore agrees with the experiments described above concerning the negative effects of thyroid excess in depressing pancreatic function.

In conclusion, it may be specially noted that the loss of nearly the whole thyroid is generally well borne by dogs, and pancreatectomy to the degree used in this experiment is compatible with indefinite longevity and liveliness at the price of some limitations of diet and body weight. The combination of the two deficiencies, however, seemed to produce a fatal cachexia. The results of animal experiments should therefore be studied very carefully before venturing upon an attempt to balance an existing pancreatic deficiency by a reduction of thyroid tissue in human patients.

Dog D4-45.—A yellow male mongrel aged 1 year, weighing 18.4 kg. in a state of medium nutrition, was partially depancreatized on Nov. 13, 1916. The tissue removed weighed 31.2 gm. The remnant about the main duct was estimated at 2.1 gm. (1/15). Fasting until Nov. 20 was then necessary to check glycosuria. A diet of lean beef and suet was next built up very gradually. At first 100 gm. meat sufficed to cause glycosuria, requiring fasting to stop it. By Dec. 29 the body weight had been reduced to 13 kg., and the limit of food tolerance had been fixed at 300 gm. meat and 100 gm. suet. Repeated attempts to feed 400 gm. meat resulted in marked glycosuria. Though thin, the dog was strong and lively, and was highly intelligent and affectionate.

On Dec. 29, all thyroid tissue was removed, with the exception of such tiny shreds as were necessary to assure the survival of one large parathyroid on each side. No glycosuria or tetanic symptoms re-

sulted. The dog fasted on the day of operation and afterward was fed almost entirely on suet up to Jan. 8, merely for the incidental purpose of observing whether the double thyroid and pancreatic deficiency created any special susceptibility to acidosis. Acetone remained absent or trivial in amount, as in a normal dog.

As the dog was not catheterized, the volume of urine and its content of total nitrogen and ammonia nitrogen were subject to irregular variations. The urine was generally scanty and concentrated, but no more so than before operation. Seemingly because of the high fat diet mentioned, the ratio of ammonia to total nitrogen was high during the first few days shown in the table, but later was normal. The principal observations pertain to the influence of thyroidectomy upon the diabetes or the food tolerance, as summarized in Table IX.

The 4 days, Dec. 9 to 12, constituted a fasting period for control purposes, as mentioned below.

Following thyroidectomy, it was possible from Jan. 8 to 13 to raise the diet not only to 400 but even to 500 gm. of lean meat without glycosuria, thus decidedly surpassing the former tolerance. The increase to 600 gm. on Jan. 14 gave rise to glycosuria of 10.6 gm., which was controlled by fasting. No marked hyperglycemia was found in the blood taken mornings before feeding during the above period. Jan. 17 to 23, 400 gm. meat with 100 or 200 gm. suet was tolerated daily. After successive increases, from Jan. 27 to 29 as much as 800 gm. meat and 200 gm. suet was eaten daily without glycosuria. The plasma sugar in the morning before feeding remained low as before.

Jan. 30 to Feb. 2 was a 4-day fasting period, for comparison with the control period of Dec. 9 to 12 before thyroidectomy. The dog was catheterized at the beginning of the first day and the end of the last day. The nitrogen output fell more rapidly and reached a lower level than in the normal state, thus showing the change of nitrogen metabolism which is known to be characteristic of total thyroidectomy.

Beginning with 500 gm. meat on Feb. 3, the diet by Feb. 5 was increased to 1 kg. of meat, which caused glycosuria of 1.5 gm., together with hyperglycemia of 0.145 per cent. on the following morning. The urine and blood then remained normal on a diet of 500 gm. meat and 100 gm. suet continued to Feb. 15.

Feb. 16 to 24, the same diet was continued with addition of thyroid tablets, beginning with dosage of 12 grains and increasing to 96 grains daily. The blood sugar rose, and glycosuria of 4.75 gm. was present on Feb. 24. This glycosuria ceased immediately with no other change than omission of the thyroid feeding. On the second day also the plasma sugar had fallen to normal. For closer comparison of the periods before and during thyroid administration, the plasma sugar was followed at 2-hourly intervals on representative days after feeding the identical diet.

On Feb. 14, before thyroid treatment, the fasting plasma sugar was 0.098 per cent. The diet of 500 gm. meat and 100 gm. suet was then fed, and the plasma sugar at 2-hourly intervals thereafter was 0.125, 0.118, 0.141, 0.151, 0.163, and 0.169 per cent.

On Feb. 23, during the period of highest thyroid dosage, the morning plasma sugar was 0.132 per cent. After the same diet, the plasma sugar at 2–hour intervals was 0.172, 0.172, 0.196, 0.238, 0.263, and 0.303 per cent. The unusually slow rise of the sugar curve is presumably explained by delayed food absorption. As this delay, however, was similar on the two days but the elevation was so much greater after thyroid feeding, it may be inferred that the thyroid had an actual influence upon the assimilative power.

After discontinuance of thyroid treatment, the diet of 500 gm. meat and 100 gm. suet continued to be tolerated. Beginning March 5, additions of first 25 and then 50 gm. of bread were borne without glycosuria, but the second day with 50 gm. of bread, resulted in the excretion of 9.75 gm. of sugar. The former protein-fat diet was then resumed.

The dog had gradually lost weight notwithstanding the liberal diets, and the feces, though always well formed, had become increasingly bulky and fatty, with a strong odor of fatty acids. At the same time a change resembling myxedema or cretinism occurred. The skin became thickened, and most of the hair was lost. The animal became sluggish, stupid almost to the point of idiocy, and indifferent to everything except a greedy desire for food. No reason is known why this change should have been produced in this dog and not in any of the others used, unless it may be the fact that this dog was much younger than the others and still retained somewhat of a puppy appearance at the time of beginning the experiment.

TABLE IX.

Dog D4-45.

Date.	Weight.	Urine.					Plasma sugar mg. per 100 cc.	Remarks.
		Vol.	Sugar.	Acetone qual.	Total—N	NH_3—N		
1917–1918	*kg.*	*cc.*	*gm.*		*gm.*	*gm.*		
Dec. 9		502	5.82	Negative.	9.43	0.65	—	Not fed.
" 10		164	Faint.	"	6.74	0.46	—	" "
" 11		256	Negative.	"	5.04	0.44	—	" "
" 12	13.6	147	"	"	4.01	0.35	—	" "
Jan. 8	12.6	148	"	"	2.29	0.99	—	Fed 100 gm. meat and 200 gm. suet.
" 9		438	"	Slight.	3.46	1.58	91	" 200 " " " 200 " "
" 10	12.3	378	"	"	3.45	1.66	—	" 300 " " " 200 " "
" 11	12.3	508	"	Faint.	7.57	2.24	126	" 400 " " " 200 " "
" 12	12.0	228	"	Negative.	4.36	1.79	—	" 400 " " " 200 " "
" 13	12.1	262	"	"	5.71	1.99	125	" 500 " "
" 14		402	10.57	Faint.	8.68	1.69	—	" 600 " "
" 15	12.1	90	Faint.	Negative.	2.23	0.69	—	Not fed.
" 16	11.9	118	Very faint.	"	2.14	0.87	—	Fed 200 gm. suet.
" 17	12.0	278	Negative.	"	6.19	1.67	—	" 400 " meat and 100 gm. suet.
" 18	11.9	148	"	"	—	—	85	" 400 " " " 100 " "
" 19		201	"	"	6.85	0.46	—	" 400 " " " 100 " "
" 20	12.0	203	"	"	6.27	1.12	104	" 400 " " " 100 " "
" 21		160	"	"	6.13	0.23	—	" 400 " " " 100 " "
" 22	12.2	198	"	"	7.52	0.46	—	" 400 " " " 200 " "
" 23	12.2	182	"	"	7.43	0.33	100	" 400 " " " 200 " "
" 24	12.5	214	"	"	8.84	0.62	—	" 500 " " " 200 " "
" 25	12.7	324	"	"	13.71	0.78	—	" 600 " " " 200 " "
" 26	12.8	Urine lost.						" 700 " " " 200 " "

Jan. 27	12.7	322	Negative.	Negative.	10.48	0.30	—	Fed 800 gm. meat and 200 gm. suet.
" 28		360	"	"	10.60	0.33	—	" 800 " " " 200 " "
" 29	12.7	408	"	"	13.41	0.77	125	" 800 " " " 200 " "
" 30	12.1	302	"	"	8.61	0.73	—	Not fed.
" 31	12.1	105	"	"	3.02	0.89	—	" "
Feb. 1	12.0	110	"	"	2.97	0.39	—	" "
" 2	11.9	70	"	"	1.85	0.06	—	" "
						0.27		
" 3	11.4	238	"	"	9.86	0.37	95	Fed 500 gm. meat.
" 4	12.2	228	"	"	9.30	0.80	—	" 800 " "
" 5	11.7	378	1.50	"	16.15	0.46	120	" 1000 " "
" 6	11.7	262	Negative.	"	9.25		145	" 500 " " and 100 gm. suet.
" 7	11.4	222	"	"	8.68	0.38	—	" 500 " " " 100 " "
" 8	11.5	282	"	"	10.42	0.69	—	" 500 " " " 100 " "
" 9	12.2	282	"	"	11.09	0.69	116	" 500 " " " 100 " "
" 10	12.0	242	"	"	8.93	0.81	—	" 500 " " " 100 " "
" 11		282	"	"	12.00	0.44	—	" 500 " " " 100 " "
" 12	12.1	188	"	"	8.69	0.49	—	" 500 " " " 100 " "
" 13	11.9	240	"	"	8.92	0.55	118	" 500 " " " 100 " "
" 14		258	"	"	8.92	0.68	98	" 500 " " " 100 " "
" 15	11.7	392	"	"	13.26	—	—	" 500 " " " 100 " "
" 16	11.7	372	"	"	14.21	—	—	" 500 " " " 100 " "
								Fed same diet with 12 grains thyroid.
" 17	11.7	235	"	"	9.65	—	—	" " " " same thyroid.
" 18		196	"	"	9.33	—	—	" " " " 20 grains thyroid.
" 19	11.8	218	"	"	8.50	—	—	" " " " 32 " "
" 20	11.6	546	"	"	15.36	—	—	" " " " 48 " "
" 21	11.8	330	"	"	13.79	—	—	" " " " 72 " "
" 22	11.3	210	"	"	10.05	—	—	" " " " 96 " "
" 23	11.3	278	"	"	10.90	—	132	" " " " 96 " "

TABLE IX—*Concluded.*

Date.	Weight.	Urine.					Plasma sugar mg. per 100 cc.	Remarks.
		Vol.	Sugar.	Acetone qual.	Total—N	NH_3—N		
1917–1918	*kg.*	*cc.*	*gm.*		*gm.*	*gm.*		
Feb. 24	11.3	460	4.75	Negative.	20.10	—	178	Fed same diet. Thyroid stopped.
" 25		288	Negative.	"	12.15	—	—	" " " " "
" 26	11.3	750	"	"	11.08	—	118	" " " " "
" 27	11.5	356	"	"	13.70	—	—	" " " " "
" 28	11.5	212	"	"	9.10	—	—	" " " " "
Mar. 1		238	"	"	9.30	—	—	" " " " "
" 2		350	"	"	14.10	—	—	" " " " "
" 3	11.8	250	"	"	7.60	—	—	" " " " "
" 4		260	"	"	11.85	—	—	" " " " "
" 5	11.5	328	"	"	14.65	—	—	" " " with 25 gm. bread.'
" 6	11.4	340	"	"	11.60	—	—	" " " " 50 " "
" 7	11.6	340	9.75	"	9.15	—	—	" " " " 50 " "
" 8	11.6	428	Negative.	"	14.50	—	—	" " " Bread stopped.
" 9	11.4	396	"	"	10.55	—	123	" " " " "

March 22, the dog was found weak and cold, barely able to stand, weighing only 8.8 kg. The urine had remained negative for sugar and acetone. Blood taken from a jugular vein showed corpuscle volume (hematocrit) of 30 per cent., plasma sugar 0.038 per cent., plasma bicarbonate (Van Slyke) 40.4 volume per cent., acetone absent.

After chloroforming, the gross autopsy was negative except for emaciation. The liver weighed only 271 gm., the kidneys together 67.5 gm., the spleen 8.5 gm. The pancreas remnant, normally soft and lobulated, weighed 2.8 gm. In view of the general atrophy, this weight as compared with the estimate of 2.1 gm. at operation may be taken to represent an appreciable hypertrophy of the remnant. The remains of thyroid-parathyroid tissue on each side of the neck were not much larger than a normal parathyroid.

Microscopically, the principal viscera were normal except for atrophy. The cortex of the adrenals was normal; the medulla was normal except for vacuolation in many of the cells, a change probably associated with the extreme cachexia. The parathyroids appeared normal. The thyroid tissue accompanying them was of microscopic dimensions, and consisted chiefly of networks of cells, with very few typical vesicles and very little colloid. The pancreas remnant was free from fibrosis. The acini were normal and well filled with zymogen. Islands were normal in number, size and structure, except for a foamy vacuolation of the cytoplasm, not resembling hydropic degeneration and probably attributable to the cachexia.

Three possible criticisms of such experiments may be answered as follows:

First, the cachexia is not due to deficiencies of diet. Bone meal was given regularly to supply salts and prevent diarrhea. Normal or potentially diabetic dogs are able to thrive indefinitely on such diets.

Second, the purpose of superposing a thyroid deficiency upon a pancreatic deficiency seems to have been strictly accomplished. Parathyroid deficiency need not be considered as a factor, for as far as known two parathyroids suffice for the bodily needs, and tetany and other recognizable signs of parathyroid deficiency were absent. Also, regeneration of thyroid tissue was so slight as not to interfere with the experiments; the extreme regeneration reported by some authors was not observed.

Third, the experiment on dog D4–45 was planned to include fecal analyses for direct proof of impaired food absorption. The feces for the different periods were demarcated with carmine, saved and dried for the purpose, but circumstances prevented the analyses, as also most of the studies of lipemia which were contemplated in this animal. The gross character of the feces, together with the dog's progressive emaciation, gave sufficient indirect evidence of the impairment of absorption. Two other dogs, one normal and the other partially depancreatized, were used as controls on the identical diets with dog D4-45, and their feces also were saved but not analyzed. As they thrived and had less bulky feces, the impaired absorption and cachexia of dog D4-45 may properly be attributed to the thyroidectomy. These troubles, however, in dog D4–45 were in no way relieved by the thyroid feeding, and likewise the appearances of myxedema and the stupid behavior remained unaltered during this time.

It must be concluded that there was a slight thyroid influence upon the assimilation in this diabetic animal. The food tolerance may have been a trifle higher after than before thyroidectomy; in particular, thyroid dosage seemed to induce hyperglycemia and glycosuria in a manner not explainable by a difference in food absorption. On the other hand, the animal still remained diabetic, and the apparent slight rise of tolerance was too dearly purchased at the price of a fatal cachexia. This evident injury robs the procedure of any practical therapeutic possibilities. From the theoretical standpoint, the partial suppression of the usual diabetic symptoms by an added injury of this character by no means indicates that the thyroid is antagonistic to the pancreas or plays any specific rôle in diabetes.

REMARKS.

1. Concerning Production of Diabetes by Thyroid Excess.—Though some experiments were negative, several positive results support the conclusion that thyroid feeding may be responsible for hyperglycemia and glycosuria in dogs which are already diabetic. The feeble influence of extremely large thyroid doses is perhaps explainable by destruction or poor absorption of the thyroid hormone in the dog's intestine. For this reason it is desirable that the experiments

should be repeated by means of parenteral injections of Kendall's thyroxin, which may be expected to exert a more powerful action. In the interpretation of any such results, a careful distinction should be maintained between the causation of hyperglycemia or glycosuria and the causation of diabetes. A combination of experimental and clinical evidence seems to demonstrate that the intoxication or the elevated metabolism of thyroid excess may aggravate existing diabetes and increase the tendency to hyperglycemia, glycosuria and downward progress. The question whether simple hyperthyroidism may give rise to hyperglycemia or impairment of glucose utilization in a normal organism without pancreatic or other disturbances may be considered still doubtful.

None of these decisions concerning either the diabetic or the normal organism have any bearing whatever upon the question of the causation of diabetes by thyroid excess. The analogous situation regarding carbohydrate excess deserves emphasis. There is no doubt that sugar and starch can produce glycosuria and downward progress in diabetes; also, sufficient quantities of sugar can produce hyperglycemia and glycosuria in the normal organism; furthermore, carbohydrate is far more powerful in these respects than the thyroid has ever been shown to be. Nevertheless, the fact is recognized both experimentally and clinically that no degree of carbohydrate excess can actually cause diabetes. Attention was formerly called[56] to the demonstration that animals with just the right degree of partial pancreatectomy may be subjected to the most extreme excesses of starch and sugar for months continuously without the slightest increase of diabetic tendency, and then diabetes may result from the removal of five-tenths, two-tenths, or even one-tenth of a gram of additional pancreatic tissue. When maximal carbohydrate excess is unable to overcome the influence of one-tenth of a gram of normal pancreatic tissue (only a small part of which consists of islands), the conclusion is unavoidable that such excess can serve only to intensify an existing or latent diabetes and is powerless as a primary factor in the causation of diabetes. The same rule must be applied to the seemingly feebler influence of the thyroid hormone. There has never been the least evidence that thyroid excess can serve in any degree to cause diabetes. The experiments with animals depancreatized almost to the point of

diabetes are simple to perform. The only positive proof must consist in the production of true diabetes in an animal which was formerly not quite diabetic. If the largest thyroid dosage proves unable to neutralize the activity of the smallest fraction of a gram of pancreatic tissue, the numerous loose claims concerning a thyroid factor in the etiology of diabetes should be silenced. From the slightness of its demonstrated influence upon carbohydrate metabolism, the writer is inclined to predict a negative conclusion of such experiments upon the rôle of the thyroid in the causation of diabetes.

2. Concerning Suppression of Diabetes by Thyroid Deficiency.—The results with thyroidectomy agree with previous views that thyroid deficiency may sometimes reduce or abolish the glycosuria and hyperglycemia of diabetes. No one, however, has ever furnished the slightest evidence that reduction of sugar by this means is beneficial. It is an unphysiological form of reasoning to argue that two deficiencies may interact so as to restore normal metabolism. These experiments accomplished the purpose aimed at by surgeons who have sought to relieve diabetes by reduction of thyroid tissue, and they proved that one deficiency is merely added to the other so as to increase the injury to health and life. Thyroidectomized dogs are known to live for long periods in fair physical condition. Those with the degree of diabetes represented in dog D4-45 can live for a long or unlimited time on regulated diets which maintain a satisfactory state of nutrition. But with thyroidectomy superimposed upon diabetes, the result was a fatal cachexia which could not be halted either by thyroid feeding or by diet. It has long been known, and was further shown in Paper 4 of this series, that any serious cachexia may abolish diabetic hyperglycemia and glycosuria. No specific action, therefore, need be assumed in the cachexia resulting from thyroidectomy. When the diabetes is sufficiently severe thyroidectomy does not abolish glycosuria.

It seems necessary to reiterate the principle that diabetes is deficiency of the internal secretion of the pancreas; it is not synonymous with glycosuria or any other symptom, and reduction of glucosuria, nitrogen excreton or any other symptoms by thyroidectomy or other mutilations does not demonstrate a reduction of the severity of the

diabetes or an antagonism between the pancreas and other glands. To prove that the thyroid hormone either inhibits the function of the islands of Langerhans, or opposes the action of their internal secretion upon the bodily metabolism, it should be shown that mildly diabetic animals with an accurately determined food tolerance may be benefited by some degree of thyroid reduction, which will enable their small pancreas remnant to function more effectively. As stated, these experiments have failed to reveal such a specific relationship, and removal of thyroid tissue has not influenced diabetes unless carried to the point of a fatal cachexia. As the animal experiments are simple to perform, and as they have thus yielded either negative or injurious results, operations upon the normal thyroids of human diabetics must be considered unjustifiable.

CONCLUSIONS.

1. Thyroid excess may aggravate the symptoms of an existing diabetes, but has never been demonstrated as contributing to the actual causation of diabetes.

2. Thyroid deficiency may partially or wholly suppress diabetic glycosuria and hyperglycemia, but this effect is rationally explained as the result of injury or cachexia. There is no indication that the intrinsic severity of the diabetes is lessened or that one deficiency can neutralize another.

3. Neither the excess nor the deficiency experiments can properly be interpreted in favor of an antagonism between the pancreas and the thyroid or of a thyroid element in diabetes.

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THE FOOD REQUIREMENTS OF CHILDREN.

IV. CARBOHYDRATE REQUIREMENT.

BY L. EMMETT HOLT, M.D., AND HELEN L. FALES.

(From the Laboratories of the Rockefeller Institute for Medical Research and the Babies Hospital.)

The carbohydrate in the diet of the growing child has, as far as is known, no specific function to perform. Nevertheless, it is a very essential component of the diet. It serves mainly as a source of energy, supplying the necessary calories which are not furnished by fat and protein. In so doing, however, carbohydrate accomplishes other purposes. It is the most efficient sparer of protein, being for this purpose superior to fat. This function of carbohydrate is so well known and has been so frequently discussed in the literature that it need not be taken up in detail here. It has been accepted generally that carbohydrate is essential for complete metabolism of fat, and that in the absence of carbohydrate from the diet, or when the amount is very low, fat is incompletely burned and acidosis may result.

When digestion is normal, a reasonable intake of carbohydrate is almost, if not quite, completely utilized in the body, leaving no residue to be eliminated in stools and urine. The excess that is not immediately used for energy is stored in the form of glycogen to be used later for energy or is converted into fat and added as such to the tissues of the body.

Carbohydrate is the most economical food from a physiologic as well as a commercial point of view. Physiologically, it is economical because of its complete utilization and because the energy required for digestion, that is, the "cost of digestion," is much lower than that of fat or protein. The commercial economy in carbohydrate foods is well known. They can be produced more cheaply than other foods; they can be stored for a long period without significant deterioration; they can be shipped and marketed in a compact form and are not readily affected by changes of temperature or by exposure. Because of

these facts the market price of carbohydrate foods is much lower than that of the foods which are composed mainly of fat and protein. Table I gives the number of calories of carbohydrate which could be purchased for 10 cents at a small retail store in New York City, Feb. 1, 1922. For comparison are given the number of calories in fat and protein foods which could be purchased for the same amount at the same time and place.

TABLE I.

Calories Purchasable for Ten Cents. Feb. 1, 1922.

Articles.	Retail price.	Number of calories.
Carbohydrate Foods:		
Sugar, granulated	6 cents per pound	3,100
Karo corn syrup	20 cents per 2.5 pound can	1,970
Oatmeal	12 cents per 20 ounce package	1,930
Rice	12 cents per pound	1,360
Bread	9 cents per 15 ounce loaf	1,270
Cream of wheat	30 cents per 28 ounce package	980
Potato	5 cents per pound	770
Jam	30 cents per pound jar	530
Fat Foods:		
Butter	49 cents per pound	740
Olive oil	65 cents per pint	600
Cream, Sheffield X (includes some protein and sugar)	32 cents per half pint	240
Protein Foods (include some fat):		
Cheese, American	35 cents per pound	590
Lean beef, round	44 cents per pound	200
Eggs, fresh brown	60 cents per dozen	180
Mixed Food:		
Milk, Sheffield, Grade A	18 cents per quart	355

Carbohydrates form the greatest part of the diet of the human race at every period of life. The nursing infant receives a high proportion of carbohydrate as lactose in woman's milk, nearly one-half of the caloric value of the milk being due to the sugar. The nursing infant takes about 50 gm. lactose daily at the end of the first month and an average of 80 gm. daily during the latter part of the nursing period. Infants taking modifications of cow's milk usually receive more carbohydrate than do nursing infants. This is given because the amount of

fat in modifications of cow's milk is in most cases considerably lower than that in woman's milk. Although a higher proportion of protein is given, this does not entirely make up for the reduction in the calories furnished as fat and consequently a larger amount of carbohydrate must be supplied.

The nursing infant receives carbohydrate entirely in the form of lactose. The infant on modifications of cow's milk may take lactose entirely or part lactose and part saccharose, maltose, dextrin, or starch, or a combination of these. It is not usual for any large proportion of the carbohydrate to be given as starch during the first year. When a mixed diet is taken at least one-half of the carbohydrate is usually in the form of starch, the rest being sugars—lactose, saccharose and fructose with occasionally a certain amount of dextrin and maltose.

The amount of carbohydrate taken by children more than 1 year of age varies according to the theories of the physician and the notions of the parent and even more according to the habits and tastes of the child. The carbohydrate variation is greater than that of any other constituent of the diet. Some children habitually take large amounts of bread, others live mainly on cereals, while with still others potatoes form the predominant article of diet. Many children consume an excessive amount of sugar, taking several teaspoonfuls on cereal and in other foods and sometimes even having sugar added to the milk taken. The relative amount of sugar taken by these children is steadily increased with age, since the child's palate demands a marked degree of sweetness in all foods and will not take those which do not taste sweet. In such cases the amount of sugar consumed soon forms a surprisingly large proportion of the diet to the exclusion of the other needed constituents.

Many children take, besides the amount of granulated sugar eaten with foods, a great deal in the form of jam, marmalade, jelly or syrup. It is often forgotten that syrup and honey are practically pure carbohydrate in a concentrated form, and that jam, marmalade and jelly usually contain from 80 to 90 per cent sugar. Many a mother or physician who would think an ounce of granulated sugar excessive does not realize that an ounce of syrup or a rounded tablespoonful of jam contains practically the same amount of sugar. To these sources of carbohydrate must be added candy. There are few children who

get no candy and the majority take several pieces and not a few as much as half a pound daily. A small piece of candy is equivalent to about one teaspoonful of sugar. It is obvious that a few pieces of candy increase the carbohydrate intake appreciably.

The degree to which sugar has come to form a prominent part of the diet of the American people is a cause for serious reflection. One hundred years ago the consumption of granulated sugar averaged only 8 pounds per capita. Last year the average was 84 pounds. The amount consumed as candy is a very large item in sugar consumption. The statement was made a few years ago by an official of a corporation operating 5 and 10 cent stores that their annual sales of candy exceeded 60,000 tons.

The carbohydrate intake of more than a hundred healthy children specially studied by us is shown in Charts 1 and 2. In Chart 1 are given the values in grams of the total carbohydrate taken daily. The curves shown are those representing the grams of carbohydrate which would be required to supply 50 per cent of the total calories which are needed, according to our estimates,[1] at the different ages. Our investigations have shown that this proportion of the total calories, that is, 50 per cent, is the average taken as carbohydrate by a normal healthy child. The figures on which this average is based will be presented later.

In Chart 2 are given the values for the carbohydrate intake in grams per kilogram of body weight. The curves represent the grams of carbohydrate supplying 50 per cent of the caloric requirement per kilo.

Charts 1 and 2 illustrate the wide variation in the carbohydrate intake of healthy children. This is most evident in the chart showing grams per kilogram. There are more very high values than very low ones and several instances of an intake of more than 15 gm. carbohydrate per kilogram. Chart 1 shows that there was more consistency in the total amount of carbohydrate taken daily. There are few low values, but several exceedingly high ones. One girl, 8 years of age, took 464 gm. carbohydrate daily. One boy, 9 years of age, took 560 gm.; another aged 10, took 488 gm. and one, aged 14, took 683 gm. daily.

1. Holt, L. E., and Fales, H. L.: Am. J. Dis. Child. 21: 1 (Jan.) 1921.

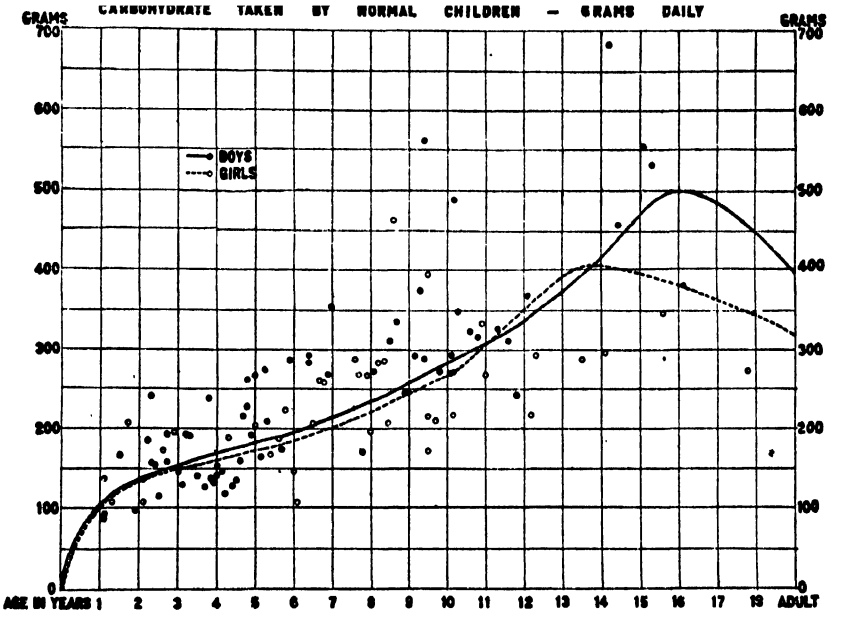


CHART 1.

The average intake according to years is shown in Table II, which gives the values for boys and girls separately and for both sexes

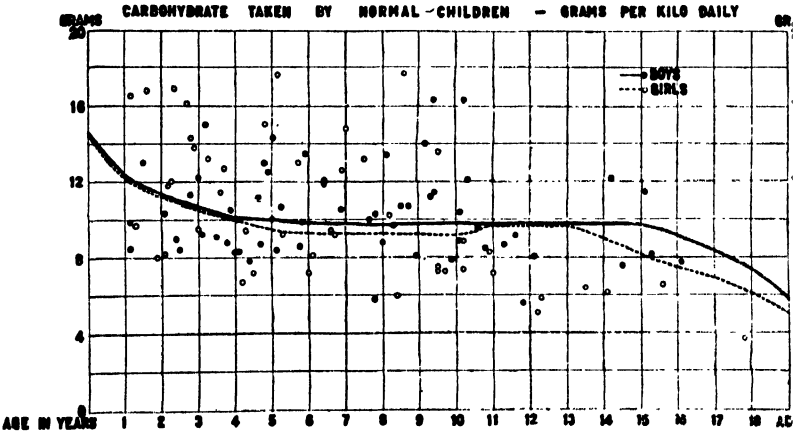


CHART 2.

grouped together. It will be seen that the total daily intake increases with fair regularity with the years, while the intake per kilogram decreases with the years. The average of all the cases, regardless of

age, is about 10 gm. per kilogram. It is interesting to note that the carbohydrate intake per kilogram of the boys aged 14 to 16 showed a marked increase over that of the boys a little younger.

The observations reported in the literature, in which the carbohydrate intake of children after infancy was ascertained, show variations quite as wide as those in our own cases. As a rule, the amount

TABLE II.

Average Carbohydrate Intake of Children. Original Observations.

Age. Years.	Boys.			Girls.			Both sexes.		
	No. of cases.	C-H intake.		No. of cases.	C-H intake.		No. of cases.	C-H intake.	
		Total gm. daily.	Gm. per kg		Total gm. daily.	Gm. per kg.		Total gm. daily.	Gm. per kg.
1–2	2	90	9.2	5	154	12.8	7	128	11.8
2–3	4	142	10.0	7	179	13.3	11	165	12.1
3–4	6	147	10.9	4	176	11.7	10	158	11.2
4–5	7	178	10.2	5	166	9.4	12	173	9.8
5–6	5	219	11.1	5	211	11.9	10	215	11.5
6–7	3	280	11.5	5	196	9.3	8	228	10.1
7–8	2	261	10.3	3	274	11.2	5	269	10.8
8–9	4	291	10.8	5	288	10.5	9	289	10.6
9–10	5	357	12.2	4	247	8.9	9	308	10.7
10–11	6	338	10.9	3	272	8.5	9	317	10.2
11–12	3	292	7.8	1	268	7.2	4	286	7.7
12–13	1	366	8.0	2	257	5.5	3	293	6.3
13–14	..	...	....	1	287	6.4	1	287	6.4
14–15	2	571	9.9	1	296	6.2	3	480	8.9
15–16	2	541	9.6	1	346	6.6	3	476	8.1
16–17	1	380	7.8	..	...	....	1	380	7.8
17–18	..	...	....	1	272	4.8	1	272	4.8

of carbohydrate per kilogram of body weight taken by young children was higher than that taken by older children. The children studied by Müller[2] had very high intakes of carbohydrate, nearly all over 10 gm. per kilogram, while several children took as much as 17 gm. The intake of carbohydrate in Camerer's cases was low, as were the other constituents. Gephart[3] found that the amount of carbohydrate taken

2. Müller, E.: Biochem. Ztschr. 5: 143, 1907.
3. Gephart, F. C.: Boston M. & S. J. 176: 17, 1917.

by the boys of St. Paul's School was much in excess of the average adult consumption, averaging more than 600 gm. daily. This represents an average of 13.7 gm. per kilogram for the youngest group of boys who averaged 13 years of age, 12.4 gm. per kilo for the group of boys who averaged 14½ years and 9.9 gm. for those averaging 16 years.

The amount of carbohydrate in the usual adult dietary is from 400 to 550 gm. daily, which represents from 5 to 8 gm. per kilogram of body weight.

Murlin[4] found that the men in the United States Army training camps took on the average 545 gm. carbohydrate daily. This is equivalent to 8.2 gm. per kilogram, the average weight being 146 pounds, or 66.5 kilograms.

From the data obtained concerning the diet taken by the normal children studied by us it was possible to calculate approximately the amounts of carbohydrate taken as sugar and as starch by these children. The values obtained are only approximate, since the relative amounts of sugar and starch used in cooking the various articles, such as cake, pudding, etc., could, in most cases, be only estimated from a knowledge of common recipes. It may be assumed, however, that our estimates are reasonably accurate.

It is surprising that the average of all the cases, regardless of age, showed that 51 per cent of the carbohydrate intake was in some form of sugar. The proportion of sugar ranged from 27 to 82 per cent, but more than two-thirds of the values were between 40 and 60 per cent. In Chart 3 are given the proportions of starch and sugar taken by each child, arranged, according to age. This shows that there is comparatively little individual variation in the distribution of the carbohydrate intake as starch and sugar. It is apparently usual in the mixed diet of children to give practically equal amounts of starch and sugar. It should be noted that the value for sugar includes not only the saccharose used in cooking or on cereals, etc., but also the fruit sugars and the lactose of the milk which was taken separately or which was used in the preparation of other foods.

Table III gives the average values according to years for the daily intake of starch and sugar.

4. Murlin, J. R., and Hildebrandt, F. M.: Am. J. Physiol. 49: 531, 1919.

CHART 3.

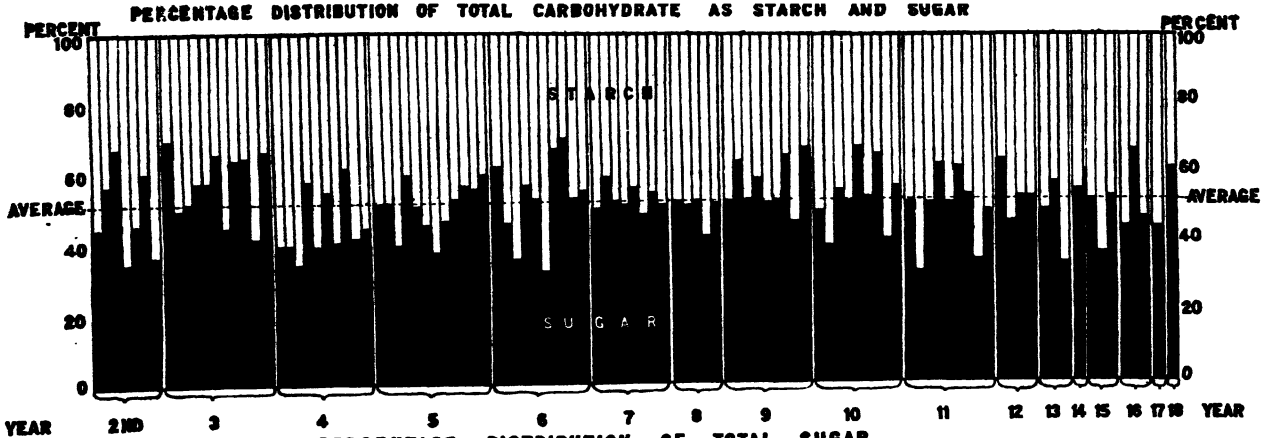
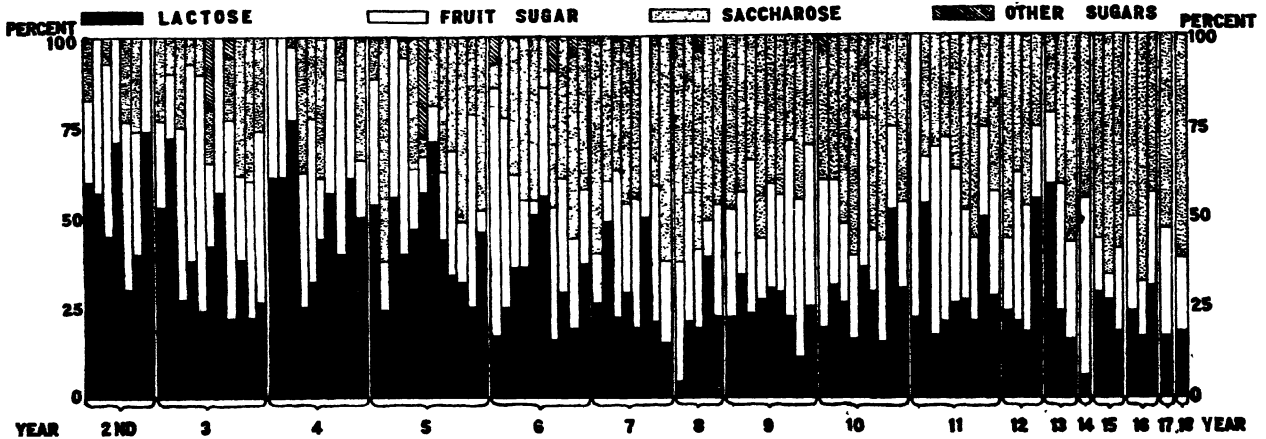


CHART 4.



It was also possible to estimate roughly the proportion of different sugars which made up the sugar intake. Some interesting observations were thus brought out. Table IV shows the averages, according to years, of the grams of lactose, saccharose and fruit sugars taken by the children studied. It will be seen that the lactose intake does not vary widely. The saccharose taken, however, shows an almost steady increase with age up to the eleventh year, with a marked increase during adolescence, the amount taken at that time being about ten times as much as the average taken by children under 4 years. It is surprising to note how much of the sugar was supplied by fruits. This

TABLE III.

Average Amounts of Starch and Sugar Taken.

Age in years.	No. of cases.	Gm. carbohydrate as		Age in years.	No. of cases.	Gm. carbohydrate as	
		Starch.	Sugar.			Starch.	Sugar.
1–2	7	65	63	10–11	9	161	156
2–3	11	71	94	11–12	4	136	150
3–4	10	85	73	12–13	3	162	131
4–5	12	86	87	13–14	1	133	154
5–6	10	101	114	14–15	3	277	203
6–7	8	111	117	15–16	3	229	247
7–8	5	137	132	16–17	1	216	164
8–9	9	128	161	17–18	1	108	164
9–10	9	149	159				

value includes all the carbohydrate in the fruit. This is largely fructose but some of it is saccharose, and there is included in some instances a small amount of starch, for example, in apples and bananas when not thoroughly ripe.

In Chart 4 are shown the percentages of the total carbohydrate taken as different sugars by each child, the values being arranged according to age.

The economic advantage of carbohydrate foods over fats and proteins has brought about a tendency to allow carbohydrate to form an excessive proportion of the modern diet. There are several important disadvantages in this procedure. The production of diabetes must be

reckoned as a possible danger, as has recently been suggested.[5] If the proper caloric intake is maintained, a very high proportion of carbohydrate necessitates a low proportion of either fat or protein. Hence, there is a possible danger of reducing the amount of fat or protein below that which is necessary for normal nutrition. When a diet high

TABLE IV.

Average Amounts of Different Sugars Taken.

Both sexes.—Average gm. daily.

Age. Years.	No. of cases.	Lactose.	Saccharose.	Fruit sugars.*	Other sugars.	Total sugars.
1–2	7	30	9	24	..	63
2–3	11	33	19	40	2†	94
3–4	10	34	12	27	..	73
4–5	12	36	26	24	1‡	87
5–6	10	35	39	37	3§	114
6–7	8	29	55	33	..	117
7–8	5	24	69	38	1†	132
8–9	9	40	64	57	..	161
9–10	9	39	76	43	1†	159
10–11	9	44	53	59	..	156
11–12	4	42	59	44	5‡	150
12–13	3	48	48	35	..	131
13–14	1	9	69	76	..	154
14–15	3	48	124	31	..	203
15–16	3	55	140	52	..	247
16–17	1	27	87	50	..	164
17–18	1	29	102	33	..	164

* This includes all sugars occurring in fruit taken; largely fructose, but includes considerable saccharose and occasionally very small amounts of starch.

† Honey (levulose and dextrose).

‡ Maltose.

§ Honey and maltose.

in carbohydrate and low in fat and protein is taken, there may result an excessive retention of water in the tissues of the body. High carbohydrate feeding leads also to an excessive deposition of fat in the body. This condition is often seen in infants fed on sweetened condensed milk or on the proprietary foods, which are chiefly carbohydrate. It

5. Micheal, J. C.: Arch. Dermat. & Syph. 2: 455 (Oct.) 1920.

is quite usual with such feeding to achieve a rapid gain in weight. Infants fed in this way, however, are usually found to have very low resistance to infection. Their tissues are high in fat and water but low in muscle. When a digestive disturbance occurs, or when an infection takes place, this unnatural weight is rapidly lost and the child often succumbs readily. A similar condition was produced experimentally in pigs by Washburn and Jones.[6] They found that young pigs fed on sweetened condensed milk put on an excessive amount of fat. The protein increase in their bodies was, however, much below normal, and the bone development was defective, the bones being only two-thirds as strong as when whole milk was fed. Table V gives a summary of some of their findings.

TABLE V.

Comparison of Growth in Seven Weeks of Six Pigs on Condensed Milk with Six Pig on Whole Milk.

Feeding.	Caloric intake daily.	Weight, gain in kg.	Fat, per cent of gain.	Protein, per cent of gain.	Breaking strength of femur in pounds.
Whole milk	1,370	11.35	9.2	15.61	362
Sweetened condensed milk	1,270	8.10	23.65	10.92	243

Whole milk contained fat, 3.3; sugar, 5.2; protein, 3.6 per cent.
Sweetened condensed milk contained fat, 1.6; sugar, 9.8; protein, 1.6 per cent.

There is a growing belief that the large and constantly increasing proportion of carbohydrate in the diet of civilized races has a very important relation to the prevalence of dental caries. It has been observed that Arctic peoples, whose diet is almost entirely fat and protein with but very little carbohydrate—and that usually in the form of starch, rarely suffer from dental caries.

Stefansson, the Arctic explorer, has stated that he himself has examined 500 skulls which were dug up from an old cemetery in Iceland of about the eleventh century. In only one skull was an imperfect tooth found and that was evidently the result of injury. He further stated that the Eskimos who live on a meat diet have perfect

6. Washburn, R. M., and Jones, C. H.: Vermont Agric. Exper. Sta. Bull. 195.

teeth, but when they come in contact with civilization and eat the foods of other races they suffer severely from dental caries.

Furthermore, archeologic researches have shown that dental caries was prevalent in ancient Egypt. More recently the discovery of a very primitive human skull in Rhodesia has shown that dental caries existed in earliest times. Both of these localities are tropical or subtropical in climate and the early diet was probably composed largely of plant foods and hence was predominantly a carbohydrate diet, a complete contrast to that taken by the Arctic peoples.

TABLE VI.

Grams of Carbohydrate Combined with One Gram Vegetable Protein.

Food.	Gm. carbohydrate per gm. protein.	Food.	Gm. carbohydrate per gm. protein.
Peanut butter	0.6	Bread	5.8
Soy beans	0.6	Rice	6.4
Walnuts	0.7	Cream of wheat	6.9
Peas	2.4	Potatoes	8.4
Lima beans	3.7	Carrots	8.4
Oatmeal	4.2		

Whether the tendency to dental caries is due to an excess of carbohydrate or to a consequent reduction of other constituents of the diet—fat, protein, mineral salts or vitamins—has not yet been established.

Of great importance, also, is the effect on digestion of a diet in which the proportion of carbohydrate is excessive. When a very large amount of carbohydrate, especially in the form of sugar, is taken into the digestive tract at one time, it is often impossible for absorption to take care of it. The consequence of this is excessive fermentation in the intestine, due to bacterial action, with the formation of carbon dioxid and organic acids, which may be very irritating to the mucous membrane of the intestine. This excess of acid usually causes a quickened peristalsis with large, loose, acid stools. The result is a decreased absorption of all the food constituents and frequently a serious diarrhea. When the diet contains an excess of carbohydrate in the form of starch, somewhat different symptoms frequently develop. There is often constipation, great abdominal distention, flatulence and colic.

Usually these disorders of digestion are acute and not very severe, yielding quickly to proper dietetic treatment. When, however, they are frequently repeated or are prolonged through lack of proper dietary adjustment, they lead to serious chronic disturbances with marked loss in weight. This condition is often difficult to control and a very great reduction in the carbohydrate of the diet is required for a long time before the normal tolerance for carbohydrate is re-established. This condition is most frequently seen in the second and third years of life.

In the discussion of the protein requirement of the child we have stated that if vegetable proteins are depended on to supply the protein need during growth very large amounts of carbohydrate are inevitably given, since vegetable proteins occur only in combination with large amounts of starch. Table VI shows the amount of carbohydrate, usually in the form of starch, which is combined and necessarily given with each gram of vegetable protein in some common foods. It will be seen from this table that nuts and soy beans are the only foods which supply much vegetable protein without including very large amounts of carbohydrate.

Although nuts have been found to be well utilized by adults without disturbance of digestion, the wisdom of giving them to children in any considerable quantity is questionable. It is not advisable to depend on them as a source of protein. It would appear from its chemical composition that the soy bean is an excellent source of vegetable protein. This vegetable, used so extensively in China, is not familiar to the American people. It has a peculiar flavor, not very palatable according to our tastes, and on that account will probably not gain any great popularity in this country, where other sources of protein are readily available.

In the other articles of food which supply vegetable protein there is so much carbohydrate combined with the protein that there is danger of serious disturbance of digestion from the excess of carbohydrate. It is evident that for this reason alone, as well as for the others given in a preceding article, it is not wise to depend on vegetable foods to supply the protein need of the child.

As has already been stated, in addition to the positive harm which may follow excessive use of carbohydrate in the diet of the child, there

must always be considered the additional disadvantage which follows this plan of feeding because of the inevitable reduction of fat and protein.

It has been shown that a definite amount of protein should be included in the diet of the growing child.[7] It seems rational to allow in the diet at least as much fat as protein.[8] The total calories needed at any age can be estimated with a fair degree of accuracy.[1] Hence, the amount of carbohydrate required is determined by subtracting from the total caloric need the calories supplied by the needed amounts of fat and protein. On this basis there should be allowed for the healthy child of average activity about 12 gm. carbohydrate per kilogram at 1 year, decreasing to between 9 and 10 gm. or about 1 per cent of the body weight at 6 years, and maintaining about this value throughout the remainder of the growth period.

If the total caloric need is raised above the average by increased muscular activity, carbohydrate may be used to provide the entire amount of extra energy, since an increase in muscular activity does not increase the needs in protein or fat above the normal.

SUMMARY.

1. Carbohydrate is a desirable and probably an essential component of the diet, although it does not have any specific function in nutrition. It forms the largest part of the diet at all periods of life.

2. Nursing infants take on the average about 12 gm. carbohydrate per kilogram of body weight daily. Artificially fed infants usually receive somewhat more than this.

3. The carbohydrate in the diet of the infant is almost all sugar, that of the nursing infant entirely lactose, that of the artificially fed infant usually a mixture of lactose with saccharose or maltose and dextrins.

4. The carbohydrate intake of more than one hundred healthy children from 1 to 18 years of age, studied by us, averaged 10 gm. per kilogram. Of this 51 per cent was sugar, including lactose, saccharose and fructose, and 49 per cent was starch.

7. Holt, L. E., and Fales, H. L.: Am. J. Dis. Child. 22: 371 (Oct.) 1921.
8. Holt, L. E., and Fales, H. L.: Am. J. Dis. Child., to be published.

5. Carbohydrate is more economical than fat or protein, both physiologically and commercially. Because of this latter advantage there is a growing tendency to increase the proportion of carbohydrate in the diet beyond the amount which is desirable.

6. When a very large proportion of the food is in the form of carbohydrate, the intake of fat or of protein or of both is likely to be less than the normal nutritive need of the body.

7. A diet excessive in carbohydrate leads to an abnormal deposition of fat without a corresponding increase in muscle development. Children taking such a diet have feeble resistance to infection.

8. There is evidence that a relationship exists between the high proportion of carbohydrate in the modern diet and the prevalence of dental caries.

9. Definite digestive disturbances chiefly intestinal, may be produced when the carbohydrate in the diet is excessive. There may result increased fermentation with loose acid stools or constipation with flatulence and abdominal distention. When long continued these disturbances are very difficult to control.

10. Carbohydrate furnishes the calories needed in the diet which are not supplied by the requisite amounts of fat and protein.

11. It seems rational to allow in the diet of the child of average activity about 12 gm. carbohydrate per kilogram of body weight at one year, decreasing the amount to about 10 gm. per kilogram at 6 years and maintaining it at this value throughout the remainder of the growth period.

12. An increase in the total caloric need because of increased activity may be supplied by carbohydrate alone.

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A METHOD FOR THE PREPARATION OF CRYSTALLINE OXYHEMOGLOBIN.

BY MICHAEL HEIDELBERGER.

(From the Hospital of The Rockefeller Institute for Medical Research.)

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Available methods for the preparation of crystalline oxyhemoglobin may be divided into two groups, in the first of which alcohol or ether, or both, are used as aids to crystallization, and in the second of which the use of these solvents is avoided. Possible objections to the use of alcohol were early pointed out by Hüfner (1), and Mayet (2) emphasized the advantages of "benzine" over ether. Although these workers, as well as Bohr (3) and Torup (4), made preparations in which the use of either or both of these substances was avoided, the first systematic attempt to work out a method without their aid seems to have been the recent one of Dudley and Evans (5). Their procedure involves pressure dialysis of the washed red cells of horse blood, crystallization of the oxyhemoglobin in the dialysate by oxidation, and recrystallization of the product from water by reduction *in vacuo* at 37° and subsequent oxidation. Data as to the yield and purity of the product are not given.

In the hope of obtaining fairly large amounts of oxyhemoglobin in the highest possible state of purity, the Dudley and Evans method was first employed, using a modification suggested by Adolph and Ferry (6); namely, final dialysis against water saturated with carbon dioxide. The oxyhemoglobin obtained in this way crystallized as large plates, instead of the needles reported by Dudley and Evans. Furthermore, the insolubility of the plates and their consequent resistance to reduction not only justified the belief that isoelectric oxyhemoglobin was being dealt with, and that Dudley and Evans were probably working with a more soluble salt, but rendered the use of the method impossible for the purpose in view, and it was abandoned in favor of a different principle.

The method now proposed depends upon observations that suspensions of washed dog or horse red cells crystallize rapidly and almost completely in the presence of toluene when saturated with carbon dioxide and oxygen, and that the resulting oxyhemoglobin may be recrystallized by solution with the aid of sodium carbonate and reprecipitated with carbon dioxide.

The use of toluene was found to hasten markedly the crystallization of the oxyhemoglobin of the corpuscles owing to its disintegrating effect on the cells themselves. While its hemolytic action is slower than that of ether, its use obviates the chief disadvantages of the latter; namely, solubility in water, and the presence of peroxides and other reactive substances which may alter oxyhemoglobin. The carbon dioxide shifts the reaction in the acid direction past the isoelectric point of oxyhemoglobin, so that the crystals obtained are oxyhemoglobin uncombined with alkali. By thus promoting the crystallization of the oxyhemoglobin the acidification also aids in the original disintegration of the corpuscles. Saturation with pure carbon dioxide would, however, drive oxygen out of the solution and change the oxyhemoglobin to the reduced form, which is too soluble to crystallize readily. In order to obviate this difficulty 1 part of oxygen was mixed in a cylinder with 4 parts of carbon dioxide for the saturation. Such a mixture may be passed through oxyhemoglobin solutions indefinitely without reduction.

Removal of the salts is accomplished by the simplified form of pressure dialysis[1] suggested by Adair, Barcroft, and Bock (7), *after* the desired number of recrystallizations has been carried out. Two recrystallizations have been deemed sufficient in this laboratory, but for many purposes the oxyhemoglobin will undoubtedly be found pure enough after the first recrystallization. On the other hand, the losses involved in each recrystallization, while appreciable, are not sufficiently large to preclude three or even four recrystallizations.

Three precautions have been found essential: (*a*) All operations are carried out in the cold, centrifugation being a possible exception if a centrifuge in a cold room is not available. (*b*) The oxyhemoglobin is not allowed to become dry, owing to the resultant change,

[1] Except that the membranes are not sterilized.

TABLE I.

Preparation No.	Volume of blood.	Total oxyhemoglobin content.	Preliminary separation in centrifuge.	First recrystallization.			Second recrystallization.			Yield.		Oxygen capacity.	Conductivity at 25° of saturated aqueous solution.*	Concentration of saturated aqueous solution.
				Volume of H_2O.	Total volume of suspension.	N Na_2CO_3	Volume of H_2O.	Total volume.	N Na_2CO_3					
	cc.	*gm.*		*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*gm.*	*per cent*	*per cent*	*mhos.*	*per cent*
Dog 8.	150	29.4	+	50	85	10–11	30	65		7.8	26.5	96.3	6.6×10^{-5}	
" 9.	295	55.5	−	50	200	6–6.5	30	80	8.5–9	13.1	23.6	100	−	
" 10.	300	45.9	−	70	160	10–11	30	90	9–10	14.4	31.4	96.3	3.9×10^{-5}	4.3
" 11.	465	76.3	+	100	230	18–22.5	70	170	18–19	29.2	38.3	99.3	5.4×10^{-5}	
" 12.	248	37.3	−	60	140	10–11	35	80	7	8.95	24.0	98.0	5.2×10^{-5}	3.9
Horse 6.	605	72.0	−	40	210	15	35	100	5–6	18.75	26.0	97.7	6.6×10^{-5}	2.7

* For the preparation of the solutions for conductivity determinations, see p. 38.

noted by Bohr (8), into a modification in which the oxygen is not reactive. (*c*) During the various manipulations on the acid side of the isoelectric point, before the final dialysis, care is taken to have an excess of carbon dioxide constantly present. If the carbon dioxide tension is permitted to fall, part of the oxyhemoglobin is redissolved as alkali salt.

The purity of the oxyhemoglobin obtained by the present method has been controlled by a determination of the ratio of the oxyhemoglobin present, as determined by Van Slyke and Stadie's procedure (9), to the total hemoglobin pigments present, determined as cyanhemoglobin by Stadie's method (10). As will be seen in Table I, preparations of 96 to 100 per cent of the theoretical oxygen capacity were obtained. The relative freedom of the product from salts was controlled by conductivity measurements of saturated aqueous solutions, the values obtained being also given in the table.

EXPERIMENTAL.

Oxalated or defibrinated dog or horse blood of known oxyhemoglobin content is centrifuged and the plasma or serum and the layer of white cells are removed. The red cells are then washed three times with chilled 0.85 per cent sodium chloride solution, after which the supernatant liquid usually gives at most only a faint haze when a test portion is boiled. The cells are then rinsed into a flask with a few cubic centimeters of water. The vessel is cooled in ice water, and a steady stream of a mixture of 4 parts of carbon dioxide to 1 part of oxygen passed in. Toluene is, meanwhile, added in amount equal to about one-seventh of the volume of corpuscles, and the mixture is stirred with the gas inlet tube until it becomes pasty. Passage of the gas is continued for a few minutes, with vigorous stirring, after which the flask is stoppered tightly with a rubber stopper and allowed to stand over night in the ice box. This is often long enough to complete the process of disintegration of the cells and crystallization of the oxyhemoglobin, but if many intact cells are still to be seen under the microscope the treatment with carbon dioxide and oxygen is repeated and the flask allowed to stand a day or two longer.

The consistency of the resulting mixture depends somewhat upon the extent to which the red cells have been packed in the centrifuge

and upon other factors which have not been determined. If the mixture is sufficiently thin it may be centrifuged with advantage in chilled tubes in a cold room, separating into an upper layer of toluene and cell fragments, an intermediate layer of clear solution, and a lower layer of oxyhemoglobin crystals. The two upper layers are poured off and the crystals drained in the ice box on a chilled porous plate, the surface layer being renewed constantly as it dries out, in order to avoid possible conversion of the oxyhemoglobin into a form in which the oxygen is less reactive. During this process a slow stream of carbon dioxide should be directed over the surface of the plate, otherwise a portion of the oxyhemoglobin will redissolve as carbon dioxide evaporates from the mixture. When drainage is as complete as possible, the oxyhemoglobin is scraped into a chilled mortar and ground to a smooth paste with sufficient ice-cold water to bring the final volume up to three to three and a half times (in cubic centimeters) the weight in grams of oxyhemoglobin present in the original blood.

In case the crude mixture of crystals, toluene, and cell fragments is too thick to permit centrifugation, the entire mass is transferred to a porous plate, using the same precautions as given above. Under these conditions the process of drainage takes much longer and cannot be carried to completion owing to the emulsion formed by the toluene. On the other hand, the product, being less compact, is easier to grind to a smooth paste with water, and the toluene and cell fragments may be removed during the first recrystallization. The final volume in this case should be kept as close as possible to that given above.[2]

The thin paste of crude oxyhemoglobin is transferred to a beaker, set in ice water, and titrated to minimum turbidity with normal sodium carbonate solution. During the addition of carbonate the mixture is stirred thoroughly, and any lumps which may remain are disintegrated. The amount of sodium carbonate necessary is greatest, of course, when the crude crystals have been thoroughly drained and contain as little as possible of the bicarbonate and salts

[2] An alternative method, which is quite satisfactory in the case of dog blood, but is very slow in the case of horse blood, is to filter the entire mass in the ice box through silk, using as large a Buchner funnel as possible, and observing the precautions given below for filtering oxyhemoglobin suspensions.

of the mother liquor. In this case the final concentration of alkali added as carbonate is approximately 0.1 N. If the toluene and cell fragments have been separated previously by centrifugation and if enough water is present, a fairly clear, deep red solution will result, but if too little water is used a crystalline precipitate of what appears to be sodium oxyhemoglobinate will remain. In this case, and also in the case in which the toluene and cell fragments are still present, the carbonate solution is added to the point of minimum turbidity, after which 1 or 2 cc. more are added in order to make sure of an excess.

The solution is next centrifuged, and any toluene and cell fragments on top are sucked off through a capillary tube, a process which can generally be accomplished without appreciable loss of the actual oxyhemoglobin solution. If loss should occur, however, the mixture which has been sucked off may be whirled again and the clear oxyhemoglobin solution added to the main portion. If enough alkali has been added and there is still a crystalline deposit in the centrifuge tubes, too little water is present, and the precipitate may be dissolved in the minimum amount of water and the solution added to the main portion. This precipitate, which is usually encountered at this point only when dog blood has been used, appears to be sodium oxyhemoglobinate, for it is readily soluble in water with a bright red color, it has a characteristic crystalline form, and, finally, yields crystals characteristic of dog oxyhemoglobin when a concentrated aqueous solution is saturated with carbon dioxide-oxygen mixture and allowed to stand in the cold. Further investigation of this salt will be undertaken.

The oxyhemoglobin solution is next chilled and a stream of the carbon dioxide-oxygen mixture passed in until crystallization *begins*, after which the flask is tightly stoppered and set in the ice box. Often within a few minutes the oxyhemoglobin has set to a solid cake of long, flat, scarlet needles in the case of dog oxyhemoglobin, and dark red, glistening, broader plates, often diamond-shaped or hexagonal, in the case of horse oxyhemoglobin.

After standing over night in the ice box the crystals are sucked off on hardened paper in a Buchner funnel (the 5 inch size is adequate for the oxyhemoglobin from 300 cc. of blood). The filtration is carried

out in the ice box, with a slow stream of carbon dioxide passing into the funnel. The surface is kept moist by renewal with a spatula as it dries out, and when this is no longer possible, a few cc. of water saturated with carbon dioxide are sucked through with the same precautions, after which the filtration is stopped. The entire process usually takes less than 1 hour.

For many purposes the oxyhemoglobin is undoubtedly sufficiently pure at this point, and in one experiment which was interrupted at this stage the amount of crystalline oxyhemoglobin recovered was 46 per cent of the amount present in the original blood, as determined by the oxygen capacity.

For further purification the recrystallization process is repeated. The crystalline cake is transferred to a chilled mortar and again ground to a smooth paste with cold water. The volume of the suspension thus obtained should be about 0.7 of that employed for the first recrystallization if the toluene and cell fragments have been initially removed by centrifugation, and from 0.4 to 0.6 as large if the removal of the upper layer was accomplished during the first recrystallization. The larger fraction will, of course, be necessary when drainage of the original crystallized cell mixture on the porous plate has been most complete. The suspension of oxyhemoglobin is dissolved with normal sodium carbonate solution, centrifuged, reprecipitated with the carbon dioxide-oxygen mixture, and collected, at every step with the same precautions as in the first recrystallization.

If salt-free oxyhemoglobin is desired, the crystals are ground with the minimum amount of cold water to a paste which will just flow easily, saturated in the cold with the carbon dioxide-oxygen mixture, transferred at once to narrow collodion dialysis bags, and dialyzed under pressure in the ice box against water saturated with carbon dioxide-oxygen mixture. Dialysis for 3 or 4 days, the carbon dioxide-oxygen-saturated water and the positions of the bags being changed daily, is sufficient to bring the conductivity down to the values given in Table I. The dialysis tubes found most suitable in this laboratory were made in 50 cc. test-tubes with one of the eminently satisfactory collodion mixtures proposed by Eggerth (11), namely, a solution of 7 gm. of "Parlodion" in 60 cc. of ether, 30 cc. of alcohol, and 10 cc. of glacial acetic acid. Dialysis under pressure was accomplished by

simply closing the ends of the tubes with tightly screwed, rubber-faced screw pinch-cocks, as proposed by Adair, Barcroft, and Bock (7).

At the end of the dialysis the contents of the bags, which still retain their crystalline structure, are sucked off in the ice box on hardened paper in a Buchner funnel, using, as before, the precaution of keeping the surface layer moist. The use of carbon dioxide at this stage is unnecessary, as the oxyhemoglobin remains sparingly soluble in the absence of alkali and salts.

The conductivity values were obtained by grinding the product in a chilled mortar with ice water, centrifuging the resulting suspension pouring off at temperatures ranging from 19° to 28°, and measuring the conductivity of the clear supernatant solution after evacuating a few times to remove any carbon dioxide present. The oxyhemoglobin content of the resulting solutions is given in Table I.

The purified oxyhemoglobin was dissolved either with the aid of sodium carbonate solution or with a sufficient excess of N/7 sodium hydroxide to bring the final concentration of alkali to 0.03 or 0.04 N, and the solution was filtered through a small, loose plug of washed cotton into a volumetric flask of appropriate size and made up to the mark. The yield of oxyhemoglobin was calculated from the oxygen capacity of the resulting solution, and varied between 23 and 38 per cent of the total originally present in the blood used. The purity of the product was determined by comparing the oxygen capacity with the total hemoglobin content as determined by Stadie's (10) methemoglobin method,[3] and the ratio of oxyhemoglobin to total hemoglobin pigments was found to vary between 96 and 100 per cent.[4]

The entire process of preparation of the dialyzed oxyhemoglobin can scarcely be completed in less than a week. On the other hand, it is not desirable to let preparations stand unduly long at the various stages of purification, for if the process is extended for much over 2

[3] It is advisable to check up the cyanhemoglobin standard at least every 2 weeks, as the color tends to deepen, even in the ice box. The color of the chilled standard also changes with rise in temperature, so that the solution should be allowed to come to room temperature before comparisons are made.

[4] The experiments recorded in this paper were all performed before the hot weather set in. Since that time yields and oxygen capacities have occasionally dropped as much as 5 per cent below the values given above.

weeks there is a noticeable diminution of the oxygen-binding power of the product. Also, when solutions saturated with the carbon dioxide-oxygen mixture are allowed to stand it is desirable to resaturate with the gas mixture at least every other day in order to compensate for leakage.

The stability to be expected of the solutions of oxyhemoglobin obtained by this method is indicated in Fig. 1, in which a steady diminution of the oxyhemoglobin content is shown amounting roughly to

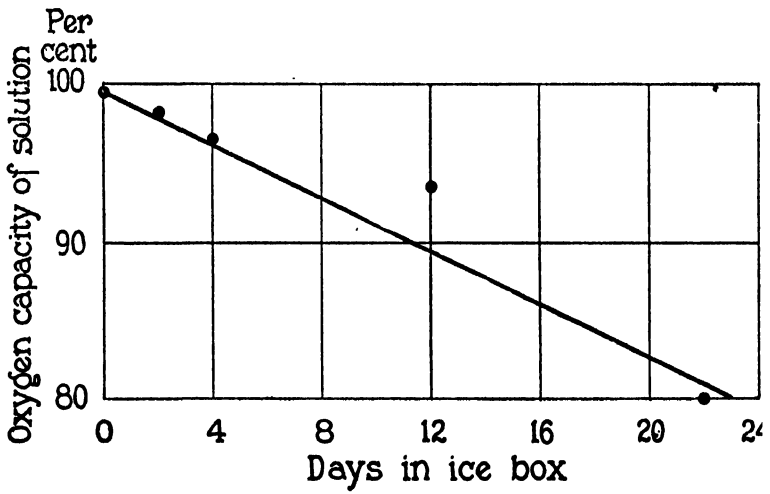

FIG. 1.

1 per cent per day. The total hemoglobin content of the original solution was 13.8 gm. per 100 cc. and the oxyhemoglobin content 13.7 gm., while the latter had fallen to 11.05 gm. at the end of 22 days.

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A STUDY OF CERTAIN PROTEIN PRECIPITANTS.

BY ALMA HILLER AND DONALD D. VAN SLYKE.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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Analytical methods for the separation of proteins from their split products are based almost entirely on the greater ease with which the proteins, as compared with their products, are thrown out of solution by various coagulants and precipitants. Some of the latter agents do not precipitate all proteins, while some of them are known to precipitate certain proteolytic products; *e.g.*, heat does not coagulate gelatin, alumina cream does not precipitate hemologbin (1); saturation with ammonium sulfate precipitates not only proteins but also some of the higher albumoses. The various precipitants are used more or or less blindly in analyses of complex mixtures such as blood and protein digests.

The present paper reports the results of an attempt to ascertain empirically but somewhat more definitely the manner in which some of these precipitants act towards the proteins and protein derivatives of blood, and of Witte's peptone as a representative mixture of intermediate products. The relative proportions of total nitrogen precipitated, and of the total nitrogen, amino nitrogen, and peptide-bound nitrogen in the filtrates have been studied with seven precipitants. The latter have been applied to blood, peptone solutions, blood plus peptone, and blood plus amino-acids. In order to simplify the amino nitrogen determinations in blood filtrates the blood urea was destroyed with urease before the precipitants were used, and the ammonia formed was removed after precipitation by vacuum distillation of the filtrates.

EXPERIMENTAL.

Preparation of Blood.—1 liter samples of ox blood were collected in large bottles containing 5 gm. each of potassium oxalate. 5 gm. of Squibb's urease were added, and the blood was allowed to stand 1

hour at room temperature. 1 per cent phenol was added and the blood was kept in the ice box, whence portions were removed as needed.

Preparation of Witte's Peptone Solution.—20 gm. of Witte's peptone were dissolved in water and diluted to a volume of 500 cc. The pH was adjusted to 7.4 and the solution was filtered. In the precipitation experiments this solution was treated as described for blood, except that the preliminary urease treatment was omitted.

Precipitation Methods.—The following methods for precipitating proteins were applied to both ox blood and Witte's peptone:

1. Colloidal Iron and Heat (2).—Before using colloidal iron for precipitating the proteins from whole blood a preliminary test was made to determine the amount of iron required. The procedure was that previously employed by Van Slyke, Vinograd-Villchur, and Losee (2) for plasma, except that we have used sodium sulfate as electrolyte instead of magnesium sulfate. Merck's dialyzed iron (5 per cent Fe_2O_3) and a 20 per cent solution of sodium sulfate were used. The procedure was the following: Into beakers were measured 20 cc. portions of water and 2 cc. portions of ox blood. The contents were heated to boiling and colloidal iron solution was added drop by drop in the amounts designated in Table I. After a few seconds boiling the sodium sulfate solution was added as indicated in the table, and the whole was thrown onto a folded filter. The results are given in Table I.

The proportions used in No. 3 seemed satisfactory for small amounts of blood, but where large volumes were used a slightly cloudy filtrate was obtained. The larger proportions of colloidal iron indicated by No. 2 were apparently required for complete precipitation.

The colloidal iron was used in routine experiments as follows: In a large beaker were mixed 300 cc. of water and 50 cc. of the Witte's peptone solution, or of ox blood which had been treated with urease as described. The mixture was heated to boiling, then 50 cc. of colloidal iron were added drop by drop with stirring. The boiling was continued for a few seconds, while 25 cc. of 20 per cent sodium sulfate were added. The mixture was allowed to cool, then washed into a 500 cc. volumetric flask, and diluted to the mark. It was filtered through a dry folded filter; 250 cc. of the filtrate were measured into a distilling flask, made alkaline to phenolphthalein with sodium hydroxide solution, and concentrated

in vacuo to about 10 cc. The residue was neutralized with acetic acid, and diluted to 25 cc. This solution was analyzed in the following manner.

(*a*) *Total Nitrogen.*—5 cc. portions were analyzed by macro Kjeldahl, using 0.02 N acid and alkali for titration.

(*b*) *Amino Nitrogen.*—2 cc. portions were analyzed according to Van Slyke (3).

(*c*) *Peptide Nitrogen.*—To 5 cc. in a hard glass test-tube were added 5 cc. of concentrated hydrochloric acid, the tube was covered with an inverted short, wide tube, and heated for 24 hours at 100° in the steam bath. The contents were then washed into a glass evaporating dish and concentrated almost, but not quite, to dryness. The concentrated filtrates were neutralized to alizarin with 40 per cent sodium hydroxide solution and diluted to 10 cc. 2 cc. were used for amino nitrogen determinations.

TABLE I.

Behavior of Colloidal Iron as Precipitant of Whole Blood.

No.	Ox blood.	Colloidal iron solution (5 per cent Fe_2O_3).	Sodium sulfate, 20 per cent solution.	Remarks.
	cc.	*cc.*	*cc.*	
1	2	3	1.5	Filters water-clear and rapidly, but precipitate very bulky.
2	2	2	1.0	Filters water-clear and rapidly, precipitate less bulky.
3	2	1.5	0.75	Filters water-clear and rapidly, precipitate slightly less.
4	2	1.0	0.5	Filtrate yellow and cloudy, precipitate much less.

2. Tungstic Acid.—The technique followed was essentially that of Folin and Wu (4).

50 cc. of ox blood or peptone solution were measured into a 500 cc. volumetric flask to which were added 300 cc. of water and 50 cc. of a 10 per cent sodium tungstate solution,[1] and the contents were well mixed. 50 cc. of 2/3 N sulfuric acid were added, the contents were again mixed, were diluted to volume, shaken several times, and after 5 or 10 minutes were filtered through a dry folded filter. Of the filtrate 250 cc. were treated exactly as described under "colloidal iron."

The final concentration of sodium tungstate is 1 gm. per 100 cc. of final mixture, and it is indicated as "1 per cent tungstate" in Table III.

[1] Primos Chemical Company product.

In the experiments indicated in Table III as "2 per cent tungstate" the conditions were the same, except that twice as much of both tungstate and sulfuric acid were used.

In a separate experiment with Witte's peptone, of which the results are given in Table VI, only one-fifth the above amount of peptone was used, the other details being the same.

3. Trichloroacetic Acid (*5*).—Ox blood and Witte's peptone were precipitated in 2.5, 5, and 10 per cent trichloroacetic acid.

2.5 Per Cent Trichloroacetic Acid.—50 cc. of blood or peptone solution were diluted with 200 cc. of distilled water, were well mixed, then diluted gradually and with constant shaking, to a volume of 500 cc. with 5 per cent trichloroacetic acid. The mixture was allowed to stand 30 minutes, and was then filtered through a dry folded filter. Of the filtrate 250 cc. were measured into a larger beaker and boiled over a free flame for 15 minutes to decompose the bulk of the trichloroacetic acid ($CCl_3COOH = CHCl_3 + CO_2$). The solution was then made alkaline to phenolphthalein with a few drops of sodium hydroxide solution, was concentrated *in vacuo*, and was treated as described under "Colloidal iron."

5 Per Cent Trichloroacetic Acid.—50 cc. of blood or peptone solution were treated as above, except that 10 per cent trichloroacetic acid solution was added instead of 5 per cent. The mixture was allowed to stand 20 minutes. Of the filtrate, 250 cc. were diluted with an equal volume of water, in order to reduce the trichloroacetic acid concentration to 2.5 per cent; since with 5 per cent a slight but measurable hydrolysis of intermediate products may occur when the solution is boiled to decompose the acid. After the dilution the filtrate was boiled and treated like the 2.5 per cent filtrate.

10 Per Cent Trichloroacetic Acid.—50 cc. of blood or peptone solution were treated as above, except that 20 per cent trichloroacetic acid solution was added instead of 5 or 10 per cent. The mixture was allowed to stand 10 minutes. Of the filtrate 250 cc. were diluted 4-fold, and the procedure continued as above.

4. Ethyl Alcohol.—50 cc. of ox blood or peptone solution were diluted to 500 cc. with 95 per cent ethyl alcohol, allowed to stand 24 hours, and then filtered through a dry folded filter. To the filtrate 0.5 cc. of saturated alcoholic solution of zinc chloride was added to precipitate the last traces of protein (6). The solution was well mixed, allowed to stand for a few moments, and again filtered. 250 cc. of the filtrate were made alkaline with sodium hydroxide and concentrated *in vacuo* to a small volume. A little water was added and the solution was again concentrated to drive off the last traces of alcohol, so that the latter would not interfere with the subsequent amino nitrogen determination. The solution was then analyzed as described under "Colloidal iron."

5. Metaphosphoric Acid (*7*).—The metaphosphoric acid was prepared according to the method of Folin (8) and a 25 per cent solution was made up just before

TABLE II.

Properties of Precipitates and Filtrates Obtained with Different Blood Precipitants.

Precipitant.	Relative volume of precipitate.	Appearance of filtrate.	Rate of filtration.	Volume of filtrate.	pH of filtrate.
				cc.	
Colloidal iron.	Very bulky.	Water-clear.	Rapid.	285	6.4
Tungstic acid.	Largest.	" "	Slowest.	340	5.1
Trichloroacetic acid.					
2.5 per cent.	Very bulky.	Water-clear.	Moderately rapid.	395	1.0
5.0 " "	Less bulky.	" "	Very rapid.	435	1.0
10.0 " "	Very small.	" "	Most rapid.	447	1.0
Alcohol.	Very bulky.	Yellow.	Moderate.	370	6.0
Metaphosphoric acid.	Moderately bulky.	Water-clear.	Very slow but faster than tungstic acid.	405	2.1
Picric acid.	Small.	Clear.	Moderately rapid.	430	2.2
Mercuric chloride.	Bulky.	Water-clear.	" "	225 (Total 300)	4.4

using. Into a 500 cc. volumetric flask were measured 200 cc. of water, 50 cc. of ox blood, or of peptone solution, and 30 cc. of the 25 per cent solution of metaphosphoric acid. The contents were well mixed and allowed to stand 1 hour, then diluted to volume with water and filtered through a dry folded filter. The remaining procedure was the same as that described under "Colloidal iron."

6. Picric Acid.—50 cc. of blood were diluted to 500 cc. with saturated aqueous picric acid solution, allowed to stand 25 minutes, and filtered. 250 cc. of the

TABLE III.

Precipitations of Witte's Peptone.

Precipitant.	Peptone in 100 cc. of precipitation mixture.	Peptone N in 100 cc. of precipitate mixture.	Percentage of original peptone N in filtrate as				Volume of filtrate from 100 cc. of mixture.	pH of filtrate.
			Total filtrate N.	Amino N.	Peptide N.	Undetermined N.		
	gm.	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*cc.*	
None	0.400	0.0584	100.0	10.4	62.5	27.1	100	7.4
Trichloroacetic acid.								
2.5 per cent	0.400	0.0584	85.4	10.5	52.3	22.6	91	>1
5 " "	0.480	0.0117		10.5	51.5			
5 " "	0.400	0.0584	77.9	9.7	46.3	21.9	92	>1
10 " "	0.400	0.0584	62.5	9.4	38.1	14.9	94	>1
HPO_3	0.400	0.0584	67.8	8.3	40.4	19.1	84	1.8
$HgCl_2$	0.400	0.0584	71.2	8.2	38.9	24.3	83	4.7
Colloidal iron	0.400	0.0584	55.2	7.0	29.7	18.5	66	3.6
Picric acid	0.400	0.0584		5.3	19.0		80	
Alcohol	0.400	0.0584	29.4	4.8	21.3	4.6	88	5.7
Tungstate.								
1 per cent	0.080	0.0117		5.3	26.7			
2 " "	0.080	0.0117		5.3	27.9			
2 " "	0.400	0.0584		4.6	17.6			
1 " "	0.400	0.0584	26.7	4.0	16.2	6.5	89	2.8

filtrate were treated as described under "Colloidal iron," the final dilution being to 50 cc. instead of 25, in order to avoid separation of an inconvenient bulk of picrate crystals.

7. Mercuric Chloride.—This precipitant was used essentially according to the technique of Gettler and Baker (9). Into a 500 cc. Erlenmeyer flask were measured 50 cc. of blood, or peptone solution, 50 cc. of water, 100 cc. of 5 per cent hydrochloric acid, and 100 cc. of 5 per cent mercuric chloride, making a total volume of 300 cc. The solution was well mixed and filtered. 150 cc. were

treated with hydrogen sulfide, and the mercuric sulfide was filtered and washed. The combined filtrate and wash water were concentrated *in vacuo* to remove hydrogen sulfide. The contents of the flask were diluted with water, made alkaline to phenolphthalein with sodium hydroxide solution, and the procedure was continued as described under "Colloidal iron."

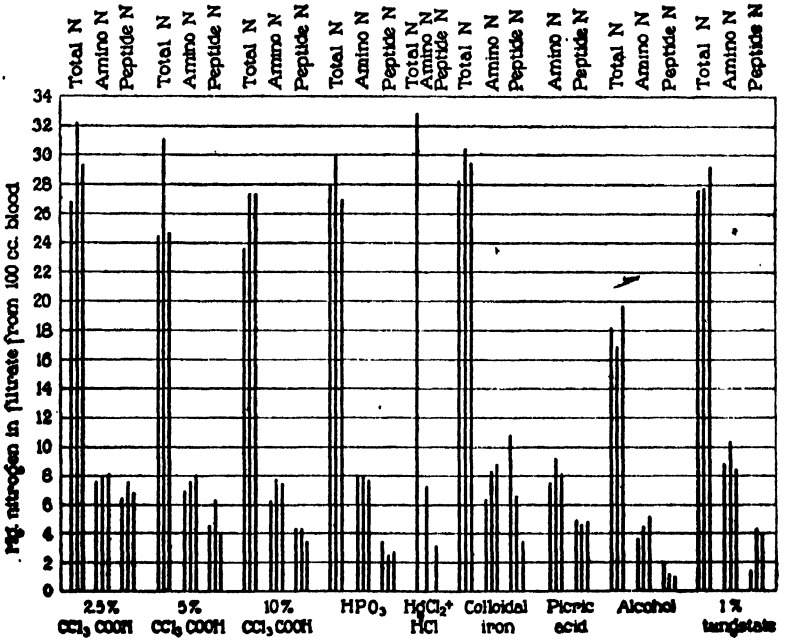

FIG. 1. Nitrogen in the filtrates from three samples of ox blood. The results from Bloods 1, 2, and 3, respectively, are indicated by three lines in order from left to right, in the case of each precipitant, except $HgCl_2$, which was tested only with Blood 2. For picric acid the total nitrogen figures are omitted, since the nitrogen content of the precipitant renders its filtrates unsuited for the Kjeldahl estimation.

The results obtained with three samples of ox blood are shown by Fig. 1, those with Witte's peptone by Fig. 2 and Table III. The figures for total nitrogen in blood filtrates were obtained after the urea had been removed, and therefore represent the non-protein, non-urea nitrogen.

Amino-Acids Added to Blood.—A solution of mixed monoamino-acids made from the phosphotungstic acid filtrate from hydrolyzed casein was added to blood, so that each 100 cc. of ox blood contained an

TABLE IV—*Concluded.*

Precipitant.	Amino nitrogen per 100 cc.				Peptide nitrogen per 100 cc.			
	Blood.	Blood + amino-acids.	Recovered.*	Added.	Blood.	Blood + amino-acids.	Recovered.*	Added.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Picric acid	9.23	30.40			4.27	6.34		
	9.29	30.85			5.19	6.75		
Average	9.26	30.63	21.37	22.00	4.73	6.55	+1.82	1.0
Alcohol	5.44	21.36			1.37	1.09		
	5.84	21.85				1.58		
Average	5.64	21.61	15.97	22.00		1.34	−0.03	1.0
Tungstic acid	9.63	31.22			5.03	4.78		
	9.98	31.25			3.52	5.60		
Average	9.81	31.24	21.43	22.00	4.28	5.19	+0.91	1.0

products as are in the filtrate, the following experiment was performed in order to test the point. The experiment was made with the filtrate from Witte's peptone rather than from blood, because the intermediate products are much more abundant in the peptone filtrate.

25 cc. portions of a 20 per cent solution of Witte's peptone were precipitated with equal volumes of 5, 10, and 20 per cent trichloroacetic acid. 3 cc. of each filtrate were neutralized with sodium hydroxide and diluted to a volume of 10 cc. 30 cc. of each filtrate were boiled in an open beaker over a free flame for 15 minutes to decompose the trichloroacetic acid, and were then diluted to 100 cc.

The amino nitrogen contents of the solutions were determined. The results as recorded in Table V showed no measurable hydrolysis as the result of boiling the peptone with trichloroacetic acid in a concentration of 2.5 per cent, but did show measurable hydrolysis by 5 and 10 per cent trichloroacetic acid.

Effect of Precipitation Time on the 5 Per Cent Trichloroacetic Acid Method.—In order to discover whether long standing after precipitation with 5 per cent trichloroacetic acid altered the results, the effect was

tested both on blood and on Witte's peptone solution, the mixtures being allowed to stand 15 minutes and 24 hours, respectively, before filtration. The results are shown in Table VII. A very slight transformation of peptide to amino nitrogen may have occurred during the longer period, but the change hardly exceeds the experimental error.

TABLE V.

Precipitation of Blood Plus One-Fifth Its Volume of 4 Per Cent Witte's Peptone.

Method.	Amino nitrogen.				Peptide nitrogen.			
	In filtrate from 100 cc. blood.			In filtrate of peptone precipitated in absence of blood.*	In filtrate from 100 cc. blood.			In filtrate of peptone precipitated in absence of blood.*
	Blood.	Blood + 1/5 volume 4 per cent peptone.	Recovered peptone amino N.		Blood.	Blood + 1/5 volume 4 per cent peptone.	Recovered peptide N of peptone.	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Trichloroacetic acid, 5 per cent.....	7.23	20.33			5.42	64.47		
	7.23	20.73			5.30	62.97		
Average.....	7.23	20.53	13.30	12.65	5.36	63.72	58.36	60.10
Tungstate, 1 per cent	10.24	16.80			9.69	39.40		
	10.38	16.85			7.54	38.95		
Average.....	10.31	16.82	6.51	6.20	8.61	39.17	30.56	31.10

* These figures are calculated from Table III.

TABLE VI.

Hydrolytic Effect of Boiling Filtrate from Peptone Solution with 2.5, 5, and 10 Per Cent Trichloroacetic Acid.

Concentration of trichloroacetic acid.	Amino N per gram of peptone.			
	Filtrate not boiled.	Filtrate boiled 15 minutes	Increase.	
per cent	*mg.*	*mg.*	*mg.*	*per cent*
2.5	13.66	13.60	0.00	0.0
5.0	12.53	13.17	0 64	5.1
10.0	10.16	11.45	1.29	12.7

Constancy of Results by the Trichloroacetic Acid Method.—In order to determine the limit of constancy in this method, several precipitations of the same blood were made with 2.5 and 5 per cent trichloroacetic acid. The results are shown in Table VIII.

TABLE VII.

Effect of Precipitation Time with 5 Per Cent Trichloroacetic Acid.

Solution.	Precipitation time.	N in filtrate from 100 cc. solution.	
		Amino N.	Amino N + peptide N.
	hrs.	*mg.*	*mg.*
Witte's peptone, 4 per cent.....	½	64.8	335
		64.2	335
" " 4 " "	24	66.4	333
		64.5	335
Ox blood.....	½	7.93	12.54
		7.99	12.95
" "	24	8.15	12.20
		8.50	13.18

DISCUSSION OF RESULTS.

Results with Peptone.—From the results with Witte's peptone it appears that tungstic acid and picric acid are distinguished by the relative completeness with which they precipitate protein intermediate products, without precipitating amino-acids. Trichloracetic acid on the other hand, particularly in solutions more dilute than 5 per cent, permitted nearly all of these products to pass into the filtrate.

It appears, therefore, that trichloroacetic acid is especially fitted for use with solutions of partially digested proteins when it is desired to remove the proteins, and to regain in their filtrates not only the amino-acids, but also a maximum proportion of the intermediate products such as "albumoses" and "peptones." Tungstic and picric acids appear better fitted for experiments in which it is desired to precipitate the intermediate products as completely as possible.

Alcohol behaves toward Witte's peptone like tungstic and picric acids, but for reasons discussed below, is not a desirable precipitant for quantitative work.

TABLE VIII.

Constancy of Results with Ox Blood by Precipitation with 2.5 and 5 Per Cent Trichloroacetic Acid.

Precipitant.	N in 100 cc. blood.				Deviation from average of 4 filtrates.			
	Total N.*	Amino N.	Amino N + peptide N.	Peptide N.	Total N.	Amino N.	Amino N + peptide N.	Peptide N.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Trichloroacetic acid, 2.5 per cent	31.14	9.99	18.68					
	30.75	10.09	18.46					
Average	30.95	10.04	18.57	8.53	+0.10	+0.26	−0.48	−0.74
Trichloroacetic acid, 2.5 per cent	31.30	9.81	18.94					
	31.58	9.81	19.16					
Average	31.44	9.81	19.05	9.24	+0.59	+0.03	±0.00	−0.03
Trichloroacetic acid, 2.5 per cent	30.47	9.64	19.50					
	30.40	9.56	19.32					
Average	30.44	9.60	19.41	9.81	−0.41	−0.18	+0.36	+0.54
Trichloroacetic acid, 2.5 per cent	30.20	9.64	19.16					
	30.90	9.72	19.13					
Average	30.55	9.68	19.16	9.48	−0.30	−0.10	+0.11	+0.21
Average of 4 filtrates	30.85	9.78	19.05	9.27				
Trichloroacetic acid, 5 per cent	26.60	9.67	15.45					
	26.78	9.52	15.32					
Average	26.69	9.60	15.39	5.79	−0.74	+0.08	+0.48	+0.40
Trichloroacetic acid, 5 per cent	27.59	9.64	14.27					
	27.59	9.63	14.36					
Average	27.59	9.64	14.32	4.68	+0.16	+0.12	−0.59	−0.71
Trichloroacetic acid, 5 per cent	27.48	9.44	14.59					
	27.30	9.33	14.86					
Average	27.39	9.39	14.73	5.34	−0.04	−0.13	−0.18	−0.05
Trichloroacetic acid, 5 per cent	27.88	9.51	15.25					
	28.20	9.36	15.14					
Average	28.04	9.44	15.20	5.76	+0.61	−0.08	+0.29	+0.37
Average of 4 filtrates	27.43	9.52	14.91	5.39				

* The total nitrogen figures represent the total nitrogen of the filtrates from blood from which the urea had previously been removed, and represent, therefore, the non-protein, non-urea nitrogen.

Metaphosphoric acid, colloidal iron, and mercuric chloride are intermediate between trichloroacetic acid and tungstic acid in the completeness with which they precipitate the intermediate products of Witte's peptone.

Results with Blood.—The average figures obtained with the different precipitants are given in Table IX.

All the precipitants used appear to remove the blood proteins completely. The completeness of the removal is indicated by the lack of high and irregular figures for the total filtrate nitrogen, and in particular for the peptide nitrogen, such as would have been ob-

TABLE IX.

Average of Results Obtained with Three Ox Bloods.

Precipitant.	Nitrogen per 100 cc. of blood.		
	Total non-protein, non-urea N.	Amino N.	Peptide N.
	mg.	*mg.*	*mg.*
Tungstic acid	28.1	9.2	4.1
Picric acid	*	8.3	4.6
Metaphosphoric acid	28.3	7.9	3.9
2.5 per cent trichloroacetic acid	28.8	7.9	7.0
Colloidal iron	29.4	7.8	†
5 per cent trichloroacetic acid	26.7	7.5	4.9
10 per cent trichloroacetic acid	26.1	7.1	4.6
Alcohol	18.2	4.9	1.4

* Not determined because of nitrogen content of precipitant.

† Not averaged because of inconsistency of results.

tained had even slight proportions of the relatively immense amounts of protein nitrogen present escaped precipitation.

Of the amino nitrogen naturally present in blood, all of the precipitants except alcohol permitted similar though not exactly equal amounts (8 ± 1 mg. per 100 cc.) to pass into the filtrates. In the filtrates from alcohol only about two-thirds as much amino nitrogen was found as in the filtrates from the precipitants used in aqueous solution. Mixed monoamino-acids from hydrolyzed casein added to blood were recovered with approximate completeness in all the filtrates except those from alcohol and metaphosphoric acid (Table IV), from which were

recovered 73 and 89 per cent, respectively. Apparently when alcohol is used as a precipitant of the blood, about 30 per cent of the free amino-acids present are adsorbed by the coagulated proteins.[2] Our findings in this respect agree with those of Bock (13).

From the peptide nitrogen data it is evident that, unlike Witte's peptone, the bloods examined contained no appreciable amounts of intermediate products precipitated by picric and tungstic acid, but not by 5 or 10 per cent trichloroacetic or metaphosphoric acid. All five of these precipitants yielded nearly the same peptide nitrogen. The bloods did, however, show 2 to 3 mg. of peptide nitrogen per 100 cc. precipitable by the above reagents, but not by 2.5 per cent trichloroacetic acid.

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[2] Van Slyke and Meyer (10) recovered relatively large amounts of added alanine almost completely from the alcoholic filtrate of dog's blood, but with the smaller concentrations of the mixed amino-acids normally present the proportion adsorbed is too great to permit quantitative recovery. The results obtained by ourselves and other authors indicate, however, that a fairly constant fraction of the total amino-acid nitrogen, *viz.* about two-thirds, is regained in the alcohol filtrate, and that this fraction is sufficiently constant to validate the conclusions drawn from comparative results in physiological experiments such as those of Van Slyke and Meyer (10), Folin (11), and Zunz (12).

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THE DETERMINATION OF THE THREE DISSOCIATION CONSTANTS OF CITRIC ACID.

BY A. BAIRD HASTINGS AND DONALD D. VAN SLYKE.

(From the Hospital of The Rockefeller Institute for Medical Research.)

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Although the first dissociation constant of citric acid has been found by conductivity to be 8.2×10^{-4} by Walden,[1] and 8.0×10^{-4} by Walker[2] at 25°, and by pH determinations 7.9×10^{-4} by Enklaar[3] at 18°, the values of the second and third dissociation constants have not been available. From the data obtained by the electrometric titration of citric acid, and from formulas developed by Van Slyke in a previous paper,[4] we have calculated the three dissociation constants of the acid.

The electrometric titration of 0.1 M citric acid with 1.0 N NaOH was carried out in a titrating electrode vessel recently described by Hastings.[5] To check these determinations the pH values of citric acid-sodium citrate mixtures, 0.1 M with respect to the citrate radical, were also determined in a Clark cell. These determinations were carried out at 20 ± 0.1°. Table I and Fig. 1 show the results.

The calculations were performed in the following way. The equation for the dissociation of a weak acid in the presence of its alkali salt, at pH ranges less than 11, is No. 31 of a previous paper;[4] *viz.*,

$$K_a' = \frac{[H^+](B + [H^+])}{C - (B + [H^+])},$$

where B is the amount of alkali added to the acid Ha, C is the concentration of the acid, γ the degree of dissociation of the salt Ba into

[1] Walden, P., *Z. physik. Chem.*, 1892, x, 568.
[2] Walker, J., *J. Chem. Soc.* 1892, lxi, 708.
[3] Enklaar, J. E., *Z. physik. Chem.*, 1912, lxxx, 617.
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[5] Hastings, A. B., *J. Biol. Chem.*, 1921, xlvi, 463.

TABLE I.

Electrometric Titration of Citric Acid with NaOH. Solutions 0.1 M with Respect to Citrate.

NaOH	pH*	$[H^+]$	NaOH	pH	$[H^+]$	NaOH	pH	$[H^+]$
mols per liter			*mols per liter*			*mols per liter*		
0.0000	2.06	8.70×10^{-3}	0.1578	4.46	3.47×10^{-5}	0.2821	6.17	6.76×10^{-7}
0.0197	2.51	3.09×10^{-3}	0.1775	4.69	2.04×10^{-5}	0.2841	6.25	5.62×10^{-7}
0.0395	2.88	1.32×10^{-3}	0.1973	4.94	1.15×10^{-5}	0.2861	6.32	4.78×10^{-7}
0.0592	3.14	7.25×10^{-4}	0.2170	5.18	6.61×10^{-6}	0.2880	6.39	4.07×10^{-7}
0.0790	3.42	3.80×10^{-4}	0.2368	5.42	3.80×10^{-6}	0.2900	6.54	2.88×10^{-7}
0.0987	3.67	2.14×10^{-4}	0.2564	5.70	1.995×10^{-6}	0.2920	6.58	2.63×10^{-7}
0.1183	3.98	1.05×10^{-4}	0.2762	6.06	8.71×10^{-7}	0.2939	6.83	1.48×10^{-7}
0.1381	4.20	6.31×10^{-5}	0.2782	6.07	8.51×10^{-7}	0.2959	7.17	6.76×10^{-8}
			0.2802	6.14	7.25×10^{-7}			

* Standard solution used for determining potential of calomel cell = 0.1 N HCl. pH assumed = 1.085, 20°. Gas chain consisted of Pt – H_2 – solution *X* – saturated KCl – $HgCl_2$ – Hg.

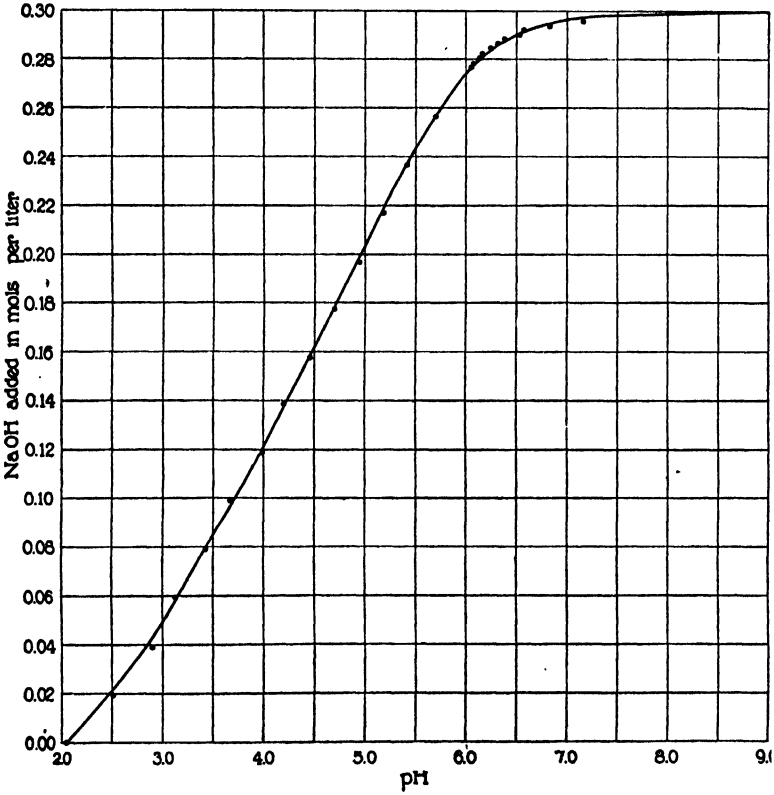

FIG. 1.

B^+ and a', and $K'_a = \frac{K_a}{\gamma}$. In solutions where the concentration of Ba is small, γ may be assumed to approach 1.0, and K'_a to approach K_a (this condition will be noted in Table III).

TABLE II.

Calculation of K'_1 from the Formula

$$K'_1 = \frac{[H^+](B + [H^+])}{C - (B + [H^+])}$$

without Correction for B_2 and B_3.

C = 0.1000

$[H^+]$	B	$B + [H^+]$	$C - (B + [H^+])$	K'_1
8.7×10^{-3}	0.0000	0.0087	0.0913	8.3×10^{-4}
3.09×10^{-3}	0.0197	0.0228	0.0772	9.1×10^{-4}
1.32×10^{-3}	0.0395	0.0408	0.0592	9.1×10^{-4}
7.25×10^{-4}	0.0592	0.0599	0.0401	10.8×10^{-4}
3.80×10^{-4}	0.0790	0.0794	0.0206	14.6×10^{-4}

TABLE III.

Calculation of K'_1 after the Correction of B_1 for B_2 and B_3 by the Formulas

$$B_2 = \frac{K'_2 C}{[H^+] + K_2} \text{ and } B_3 = \frac{K'_3 C}{[H^+] + K'_3}$$

$K'_2 = 4.1 \times 10^{-6}$

$K'_3 = 3.2 \times 10^{-6}$

C = 0.1000

$[H^+]$	Total B	B_2	B_3	B_1	$B_1 + [H^+]$	$C - (B_1 + [H^+])$	K'_1
8.7×10^{-3}	0.0000	0.0000	0.0000	0.000	0.0087	0.0913	8.3×10^{-4}*
3.09×10^{-3}	0.0197	0.0013	0.0001	0.0183	0.0214	0.0786	8.4×10^{-4}
1.32×10^{-3}	0.0395	0.0030	0.0002	0.0363	0.0376	0.0624	7.9×10^{-4}
7.25×10^{-4}	0.0592	0.0054	0.0004	0.0534	0.0541	0.0459	8.5×10^{-4}
3.80×10^{-4}	0.0790	0.0098	0.0008	0.0684	0.0688	0.0312	8.3×10^{-4}

* The value for this first figure is really K_1, rather than K'_1, since no salt is present.

Average $K'_1 = 8.3 \times 10^{-4}$

$pK'_1 = 3.08$

The values of K'_1, K'_2, and K'_3 were estimated by successive approximations. (We shall refer to the K'_a of the most strongly dissociated

acid group as K'_1, of the middle one as K'_2, and of the least dissociated as K'_3.) From the data towards the most acid and least acid ends of the titration curve (Fig. 1), first approximations of the values of K'_1 and K'_3, respectively, were made (Tables II and IV). From the approximate values of K'_1 and K'_3 thus obtained the amounts of base, B_1 and B_3, bound by the two end-carboxyls over the middle part of the curve, were calculated by Equation 10 of the previous paper;[4] *viz.*, $B = \frac{K'C}{[H^+] + K'}$. The B_1 and B_3 values thus calculated were subtracted from the total amount of NaOH added (B) to give B_2. $B_2 = B - B_1 - B_3$. From the values of B_2 thus obtained K'_2 was calculated.

TABLE IV.

Calculation of K'_3 from the Formula

$$K'_3 = \frac{[H^+][B]}{C - B}$$

without Correction for B_1 and B_2.

C = 0.1000

$[H^+]$	B	C − B	K'_3
2.63×10^{-7}	0.0920	0.0080	3.0×10^{-6}
2.88×10^{-7}	0.0900	0.0100	2.6×10^{-6}
4.07×10^{-7}	0.0880	0.0120	3.0×10^{-6}
4.78×10^{-7}	0.0861	0.0139	3.0×10^{-6}
5.62×10^{-7}	0.0841	0.0159	3.0×10^{-6}
6.76×10^{-7}	0.0821	0.0179	3.1×10^{-6}
7.25×10^{-7}	0.0802	0.0198	2.9×10^{-6}
8.51×10^{-7}	0.0782	0.0218	3.0×10^{-6}
8.71×10^{-7}	0.0762	0.0238	2.8×10^{-6}
1.995×10^{-6}	0.0564	0.0436	2.6×10^{-6}
3.80×10^{-6}	0.0368	0.0632	2.2×10^{-6}

From this value of K'_2 and the above mentioned first approximations of K'_2 and K'_3, B_2 and B_3 were estimated, in order to calculate the exact B_1, at the acid end of the curve by the equation $B_1 = B - B_2 - B_3$. From the B_1 values thus obtained, a series of consistent values for K'_1 was obtained (Table III).

The B_3 values at the alkaline end of the curve were then estimated as $B_3 = B - B_1 - B_2$, the B_1 and B_2 values being obtained from the

K'_1 and K'_2 values, found as above described. The B_3 values thus obtained yielded a series of consistent values for K'_3 (Table V).

TABLE V.

Calculation of K'_3 after the Correction of B_3 for B_1 and B_2 by the Formulas

$$B_1 = \frac{K'_1 C}{[H^+] + K'_1} \text{ and } B_2 = \frac{K'_2 C}{[H^+] + K'_2}$$

$K'_1 = 8.3 \times 10^{-4}$

$K'_2 = 4.1 \times 10^{-5}$

$C = 0.1000$

$[H^+]$	Total B	B_1	B_2	B_3	C − B	K'_3
2.63×10^{-7}	0.2920	0.1000	0.0994	0.0926	0.0074	3.3×10^{-6}
2.88×10^{-7}	0.2900	0.1000	0.0993	0.0907	0.0093	2.8×10^{-6}
4.07×10^{-7}	0.2880	0.1000	0.0991	0.0889	0.0111	3.3×10^{-6}
4.78×10^{-7}	0.2861	0.1000	0.0990	0.0871	0.0129	3.2×10^{-6}
5.62×10^{-7}	0.2841	0.1000	0.0988	0.0853	0.0147	3.3×10^{-6}
6.76×10^{-7}	0.2821	0.1000	0.0985	0.0836	0.0164	3.4×10^{-6}
7.25×10^{-7}	0.2802	0.1000	0.0983	0.0819	0.0181	3.3×10^{-6}
8.51×10^{-7}	0.2782	0.1000	0.0982	0.0800	0.0200	3.4×10^{-6}
8.71×10^{-7}	0.2762	0.1000	0.0981	0.0781	0.0219	3.1×10^{-6}
1.995×10^{-6}	0.2564	0.0998	0.0954	0.0612	0.0388	3.1×10^{-6}
3.80×10^{-6}	0.2368	0.0996	0.0917	0.0455	0.0545	3.2×10^{-6}

Average $K'_3 = 3.2 \times 10^{-6}$

$pK'_3 = 5.49$

TABLE VI.

Calculation of K'_2 from the Formula

$$K'_2 = \frac{[H^+]\ [B_2]}{C - B_2}$$

where B_2 is Calculated from

$B_2 = B - [B_1 + B_3]$

$K'_1 = 8.3 \times 10^{-4}$

$K'_3 = 3.2 \times 10^{-6}$

$C = 0.1000$

$[H^+]$	Total B	B_1	B_3	B_2	$C - B_2$	K'_2
3.80×10^{-4}	0.0790	0.0686	0.0008	0.0096	0.0904	4.0×10^{-5}
2.14×10^{-4}	0.0987	0.0798	0.0015	0.0174	0.0826	4.5×10^{-5}
1.05×10^{-4}	0.1183	0.0888	0.0030	0.0265	0.0735	3.8×10^{-5}
6.31×10^{-5}	0.1381	0.0930	0.0048	0.0403	0.0597	4.3×10^{-5}
3.47×10^{-5}	0.1578	0.0961	0.0084	0.0533	0.0467	4.1×10^{-5}
2.04×10^{-5}	0.1775	0.0977	0.0135	0.0663	0.0337	4.0×10^{-5}

Average $K'_2 = 4.1 \times 10^{-5}$

$pK'_2 = 4.39$

K'_2 was finally reestimated by using the accurate K'_1 and K'_3 values to calculate the B_1 and B_3 figures of the equation $B_2 = B - B_1 - B_3$. The B_2 values thus obtained yielded a series of consistent values for K'_2 (Table VI).

TABLE VII.

Calculation of K'_2 from the Buffer Value β_2.

$pK'_1 = 3.08$

$pK'_3 = 5.49$

pH	ΔpH	Mean pH	ΔB	$\frac{\Delta B}{\Delta pH}$	$pH - pK'_1$	$pH - pK'_3$	β_1	β_3	β_2	$pH - pK'_2$	pK'_3
3.69 3.95	0.26	3.82	0.02	0.0770	0.74	−1.67	0.0301	0.0046	0.0423	−0.49	4.31
3.82 4.08	0.26	3.95	0.02	0.0770	0.87	−1.54	0.0241	0.0063	0.0466	−0.40	4.35
3.95 4.22	0.27	4.09	0.02	0.0741	1.01	−1.40	0.0190	0.0083	0.0468	−0.39	4.48
4.08 4.35	0.27	4.22	0.02	0.0741	1.14	−1.27	0.0146	0.0112	0.0483	−0.37	4.59
4.22 4.48	0.26	4.35	0.02	0.0770	1.27	−1.14	0.0112	0.0147	0.0511	−0.30	4.65
4.35 4.59	0.24	4.47	0.02	0.0834	1.39	−1.02	0.0086	0.0184	0.0564	+0.10	4.37
4.48 4.72	0.24	4.60	0.02	0.0834	1.52	−0.89	0.0063	0.0233	0.0538	+0.23	4.37
4.59 4.84	0.25	4.72	0.02	0.0800	1.64	−0.77	0.0052	0.0288	0.0460	+0.42	4.30
4.72 4.97	0.25	4.85	0.02	0.0800	1.77	−0.64	0.0037	0.0345	0.0415	+0.51	4.34

K'_2 was also calculated from the buffer value $\frac{dB}{dpH}$. The total buffer value of the solution at any pH may be expressed as $\beta = \beta_1 + \beta_2 + \beta_3$. β may be evaluated from the titration curve by calcu-

lating $\frac{\Delta B}{\Delta pH}$. β_1 and β_3 may be calculated[6] from K'_1 and K'_3 by Equation 35; *viz.*,

$$\beta = 2.3\left(\frac{K'C[H^+]}{(K' + [H^+])^2} + [H^+] + [OH']\right),$$

the [OH'] being negligible in the present case. They may also be estimated graphically by means of Fig. 9 of the previous paper.[4]

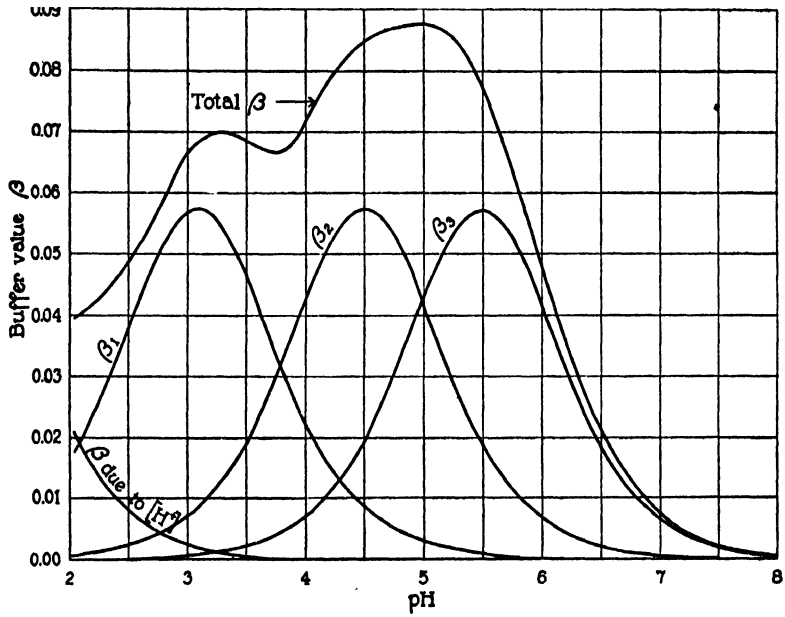

FIG. 2.

β_2 is then obtained by difference, and K'_2 calculated either from Equation 22,

$$K'_2 = [H^+]\frac{C - 0.8686\beta_M \pm \sqrt{(0.8686\beta_M - C)^2 - 0.756\beta_M^2}}{0.8686\beta_M}$$

or graphically, as described in the previous paper, from Fig. 9. That value of K'_2 is the correct one which is identical with the $[H^+]$ when $\beta_M = 0.575$.

[6] The equation numbers used in the present paper refer to the numbered equations in a former paper.[4]

Values of pK'_2 obtained by this method are given in Table VII. The value at that point where β_2 most nearly approaches its maximum of 0.0564, is $pK' = 4.37$. Other values range from 4.30 to 4.65. The agreement with the first method of calculation is, we believe, sufficiently good.

The value of the buffer effect of 0.1 M citrate, estimated from the above three pK′ values by graphic summation (as in[4] Fig. 8) are given in Fig. 2.

SUMMARY.

The methods for the calculation of the dissociation constants of weak polybasic acids recently outlined[4] have been applied to citric acid. The values of the three constants have been found to be $K'_1 = 8.3 \times 10^{-4}$, $K'_2 = 4.1 \times 10^{-5}$, and $K'_3 = 3.2 \times 10^{-6}$. The corresponding pK′ values are 3.08, 4.39, and 5.49, respectively. The value of K'_1 agrees approximately with that of K_1 found by other authors.[1,2,3] The values of K'_2 and K'_3, because of the overlapping effects of the carboxyl groups, have not been accessible by previous methods of calculation.

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THE EFFECT OF ETHER ANESTHESIA ON THE ACID-BASE BALANCE OF THE BLOOD.

BY DONALD D. VAN SLYKE, J. HAROLD AUSTIN, AND GLENN E. CULLEN.

(From the Hospital of The Rockefeller Institute for Medical Research, New York, and the Department of Experimental Medicine of the University of Pennsylvania, Philadelphia.)

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There has been disagreement in the literature concerning the effect of ether anesthesia on the acid-base balance. Menten and Crile (1915) reported a fall in the blood pH in rabbits. Caldwell and Cleveland (1917) found a consistent fall in the plasma bicarbonate as the result of ether anesthesia in patients. Carter (1920) found a similar fall in etherized dogs. If observations by Caldwell and Cleveland and Carter on blood bicarbonate could be linked with Menten and Crile's pH determinations on rabbits, the combined data, indicating lowered [$BHCO_3$] and lowered pH, respectively, could indicate nothing but an uncompensated alkali deficit.

However, Y. Henderson and Haggard (1918) in experiments on dogs showed that the blood alkali reserve, as measured by the CO_2 capacity, could be lowered by experimental hyperventilation. The latter lowers the free H_2CO_3 of the blood, with the result that as shown by Milroy (1914), an increased pH results. The results of Henderson and Haggard indicated that as a secondary effect there may also be a shift of alkali from blood to tissues, or of acid in the reverse direction. Such an effect could be explained as an attempt to lessen the increased pH resulting from abnormal lowering of H_2CO_3. The result of such a condition is indeed a lowered bicarbonate, but, it is primarily due to H_2CO_3 deficit, or CO_2 alkalosis (exemplified by Areas 2 and 3 of Fig. 1, Van Slyke, 1921, *b*), rather than to alkali deficit resulting from entrance of non-volatile acids (exemplified by Areas 6 and 9).

Hasselbalch and Lundsgaard (1912) had showed that merely tying rabbits down may result in a gradual fall in blood pH; so there ap-

peared to be some reason for being uncertain that Menten and Crile's observed fall in blood pH was due to etherization. Henderson and Haggard reported no pH determinations, but their results with ether seemed most readily interpreted by the CO_2 deficit explanation.

It appeared that a decision between the two diametrically opposed interpretations of the observed fall in blood [$BHCO_3$] could be reached only by observations in which both [$BHCO_3$] and pH were determined. In a preliminary report (1920) the present authors showed that the pH falls (in agreement with Crile and Menten) whenever it undergoes any change during ether anesthesia. Fall in bicarbonate was consistently observed.

Since the appearance of the above report Collip (1920), experimenting with etherized dogs, calculated the blood pH from the CO_2 tension of the alveolar air and the CO_2 content of the blood. He found this method not entirely satisfactory, but indicative of a fall in blood pH. Recently Atkinson and Ets (1922) have again determined both CO_2 content and pH (the latter by the Dale and Evans colorimetric dialysis method) in the blood of etherized dogs, and have found a fall in both, with recovery after discontinuance of anesthesia.

In the present paper we report the results of six representative experiments. The pH changes in some cases were determined electrometrically, in others colorimetrically, and in others by calculation from the [$BHCO_3$]: [H_2CO_3] ratio obtained by equilibration with known CO_2 tensions. The bicarbonate was determined both gasometrically and by titration.

The depth and time of anesthesia have been varied. The results concerning the acid-base change have been consistent, and we have consequently felt justified in reporting in detail only a sufficient number of experiments to indicate fairly the nature of the data obtained.

METHODS.

Animals.—Large dogs (10 to 15 kilos) were used so that large samples of blood (25 to 50 cc.) might be taken. The dogs were fed as usual the day preceding the experiment but received no food on the day of the experiment.

Bleeding.—The blood was drawn from the left ventricle (unless stated otherwise) through a 4 inch, 16 gauge lumbar puncture needle into a tube under oil. When oxalated blood was desired the tube was previously coated with neutral potassium oxalate to make 0.3 per cent. If defibrinated blood was wanted, the blood was defibrinated under oil by gentle stirring. Since it is usually impossible to avoid some hemolysis in oxalated plasma of dog's blood, it was decided in the later experiments to utilize the true serum of the blood as drawn, allowing coagulation to occur spontaneously while centrifuging.

In centrifuging for true plasma or true serum the blood was drawn directly into a centrifuge tube of the proper size, containing mineral oil. The glass delivery tube was withdrawn as the blood ran in, so that the tube was completely filled with the blood except for a layer of paraffin oil about 1 cm. deep. A 1-hole rubber stopper was inserted, with complete expulsion of the oil. The hole was closed with a glass plug and the tube centrifuged at once. After centrifuging, the glass plug was removed, and from a pipette oil was allowed to flow through the hole in the stopper as the stopper was removed. It is shown elsewhere that such precautions are necessary to prevent loss of CO_2 during centrifugation. The plasma or serum was then transferred without loss of CO_2 to Haldane sampling tubes over mercury or to tubes under oil.

Anesthesia.—The animal was anesthetized in all cases by the drop method with a few layers of gauze. This method was continued throughout the anesthesia unless otherwise stated.

Ventilation Rate.—Ventilation rate was measured in the control periods and during the early part of anesthesia by the use of a closely fitting, well greased rubber mask that fitted and enclosed the entire muzzle of the dog. A Y-tube close to the mask led to two 1 inch aluminum Siebe Gorman valves. The expired air was collected and measured in a 65 liter spirometer.

After the animal was anesthetized a cannula was tied in the trachea and connected to the Y-tube and valves. The intake of air was through a vessel containing gauze onto which ether could be dropped to maintain the anesthesia.

Equilibration of Blood or Serum with CO_2.—The blood was introduced into a partially evacuated tonometer which contained the required

amount of CO_2 and had an oxygen tension approximately atmospheric at 38°. The tonometer was then rotated in a water bath at 38° until equilibration was complete. The equilibration was either repeated or the CO_2 tensions corrected for the CO_2 taken up or given off by the blood. The blood was then transferred, with precautions to prevent loss of CO_2, to Haldane sampling tubes over mercury, or under oil to centrifuge tubes. The centrifuging was carried out with the precautions described above.

Hydrogen Ion Concentration Measurements.—Electrometric.—These determinations were made at 20° on whole blood with the Clark cell using Hasselbalch's refilling technique. Although the determinations were made with the cells present, Parsons (1919–20) has shown that the pH determined is that of the plasma.

Colorimetric.—The colorimetric pH measurements were made at room temperature with phenol red in the diluted plasma or serum by the method recently described by Cullen (1922). The correction used was −0.34, to reduce pH colorimetrically determined at 20° to that electrometrically found at 38°. This correction may subsequently be altered, but such alteration would not affect the pH changes observed.

Analytical Methods.—The carbon dioxide determinations were made usually in duplicate on 1 cc. samples by Van Slyke's method with either the fine bore constant pressure apparatus (Van Slyke and Stadie, 1921), or the constant volume apparatus (Van Slyke, 1921, *a*). In Experiment 5, in addition to the gasometric determination of the total CO_2 content, the $BHCO_3$ was determined directly by the titration method (Van Slyke, 1922, *b*).

Calculation.

In discussion of the calculations we shall use the following abbreviations: mM. for millimolar; $[CO_2]$, $[BHCO_3]$, $[H_2CO_3]$ for mM. concentration of CO_2, $BHCO_3$, and H_2CO_3, respectively; p_{CO_2} for CO_2 tension in millimeters of mercury.

In these experiments we have obtained by analysis two or more of the following data: $[CO_2]$ of the blood, plasma, or serum, as drawn from the left ventricle or femoral artery; $[CO_2]$ of the oxygenated blood,

true plasma, or serum after equilibration of the blood at 38° in tonometers at known p_{CO_2}; the pH determined electrometrically or colorimetrically on the plasma or serum as drawn, or upon the true plasma or serum after equilibration at known p_{CO_2}. Our problem has been to determine from these data the changes that have occurred, *in vivo*, in the alkaline reserve, CO_2 tension, and pH of the blood.

In Experiments 1 to 3 we have data on the $[CO_2]$ of blood, or the plasma of blood, equilibrated at various CO_2 tensions, and the $[CO_2]$ of the arterial blood, plasma, or serum, as drawn. From the $[CO_2]$ and p_{CO_2} values determined in the equilibrated blood or its true serum we have estimated the pH values of the blood plasma, and plotted them as abscissæ against the $[CO_2]$ values as ordinates. The resulting curves are almost exactly straight lines.[1] By interpolating the $[CO_2]$ values observed in the blood as drawn on these lines we have determined the pH of the blood as drawn. From the pH thus interpolated and the $[CO_2]$, the p_{CO_2} and $[BHCO_3]$ were calculated.

In constructing our $[CO_2]$, pH curves we have in each case drawn the mean straight line through the points determined on the equilibrated bloods. It appears that such a line compensates for errors in individual determinations and is a more accurate representation of the correct $[CO_2]$, pH curves than a broken line drawn through the individual points. The pH values corrected by means of the straight line graphs thus drawn are indicated in the tables as "Rectified pH"

[1] The fact that when blood pH is plotted against $[CO_2]$ straight line curves are obtained over the physiological range of CO_2 tensions was first noted by Lewis, Cotton, Barcroft, Milroy, Duften, and Parsons (1916). McLean, Murray, and Henderson (1920) found straight line curves also when they plotted pH values against $[BHCO_3]$. The approximately linear character of these curves is attributable to the, as one might say, accidental fact, that the COOH groups of hemoglobin are so arranged that the buffer value (Van Slyke, 1922, *a*) of blood over the physiological PH range is practically constant.

Both curves cannot be exactly linear, for the $[H_2CO_3]$ area which separates $[CO_2]$ from $[BHCO_3]$ is curved. However, this area above pH 7 is relatively narrow, so that it does not prevent both the $[CO_2]$ and $[BHCO_3]$ curves from approximating the linear form. Which of the two does so most closely is at present uncertain, since both appear, over the range pH 7 to 7.8 to vary from straight lines by no more than the experimental errors heretofore connected with pH and $[CO_2]$ measurements.

points. It will be noted that the rectifications fall within the limit of 0.02 pH, which may be taken as the limit of experimental error.

The calculations of pH from $[CO_2]$ and p_{CO_2} values obtained from equilibration data, and of p_{CO_2} and $[BHCO_3]$ from the determined $[CO_2]$ in the blood as drawn and the pH found by graphic interpolation, were performed by means of Hasselbalch's (1917) equation, $pH = pK' + \log \frac{[BHCO_3]}{[H_2CO_3]}$. For the above respective calculations the equation was rearranged into the following forms by steps given in another place (Austin, Cullen, Hastings, McLean, Peters, and Van Slyke, 1922).

$$\text{For whole blood, } pH = 6.20 + \log \frac{[CO_2] - 0.0300\, p_{CO_2}}{0.0300\, p_{CO_2}},$$

$$p_{CO_2} = \frac{1}{0.0300} \times \frac{[CO_2]}{10^{pH - 6.20} + 1}, \quad [BHCO_3] = [CO_2] \times \frac{10^{pH - 6.20}}{10^{pH - 6.20} + 1}.$$

$$\text{For plasma, } pH = 6.10 + \log \frac{[CO_2] - 0.0318\, p_{CO_2}}{0.0308\, p_{CO_2}},$$

$$p_{CO_2} = \frac{1}{0.0318} \times \frac{[CO_2]}{10^{pH - 6.10} + 1}, \quad [BHCO_3] = [CO_2] \times \frac{10^{pH - 6.10}}{10^{pH - 6.10} + 1}.$$

The values of pK', 6.10 for plasma and 6.20 for whole blood, are the averages determined on a number of dog bloods. They may later be corrected in the second decimal places but such correction would not significantly affect the *changes* in pH, p_{CO_2}, and $[BHCO_3]$ calculated in our experiments.

In terms of millimolar concentration, $[H_2CO_3] = \frac{\alpha CO_2 \quad p_{CO_2}}{0.0224 \times 760}$. This value is $0.0300\, p_{CO_2}$ when α_{CO_2} (the solubility coefficient of CO_2) is 0.511, as in whole blood (Bohr, 1905), while it is $0.0318\, p_{CO_2}$ when α_{CO_2} is 0.541, as in serum.

Changes in alkali reserve have been measured as changes in the bicarbonate content of the blood estimated at a given pH; *viz.*, the pH observed before ether was administered. This pH was interpolated on the pH, $[CO_2]$ graph obtained after ether, and the $[BHCO_3]$ was calculated for the point thus located. At constant pH, the change in blood $[BHCO_3]$ expresses directly the change in the excess of total base over acids other than H_2CO_3 (Van Slyke, 1921, *b*, p. 169), and

because of this advantage in definiteness of interpretation, we have chosen to measure bicarbonate changes at a given pH rather than at a given p_{CO_2}.

The mode of calculation above outlined, and used in Experiments 1, 2, and 3, is illustrated by Fig. 1, by data from Experiment 3.

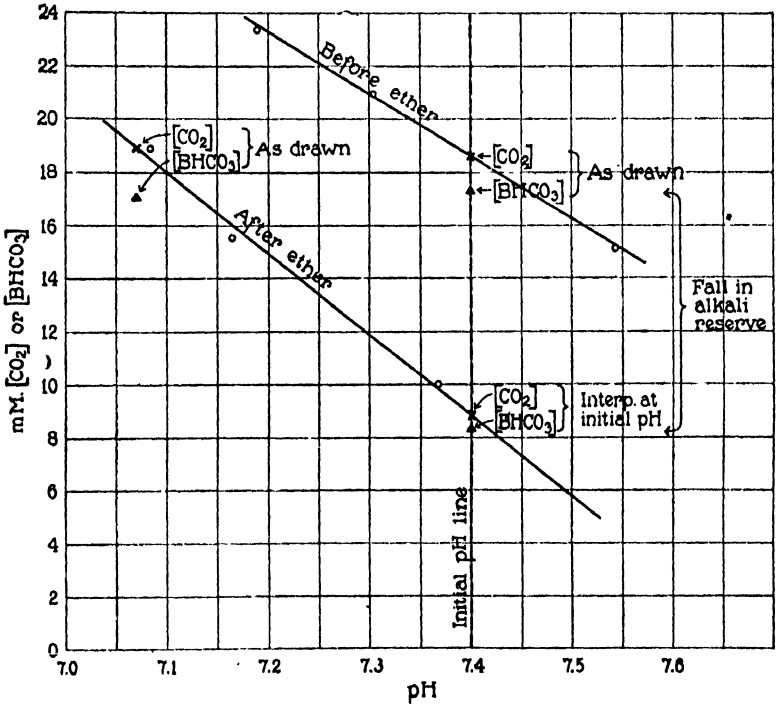

FIG. 1.

In Experiments 4, 5, and 6 the data directly determined have been the pH and the $[CO_2]$ of the blood as drawn. From these the $[BHCO_3]$ and p_{CO_2} have been calculated by the equations previously given.

Extrapolation of the $[BHCO_3]$ value of the bloods drawn after etherization to the values which those bloods would have under CO_2 tension such as would restore pH to its initial value, was necessary in order to measure the alkali reserve change at constant pH. For constructing the pH, $[BHCO_3]$ lines necessary for the extrapolation in these experiments, there were available no graphs experimentally

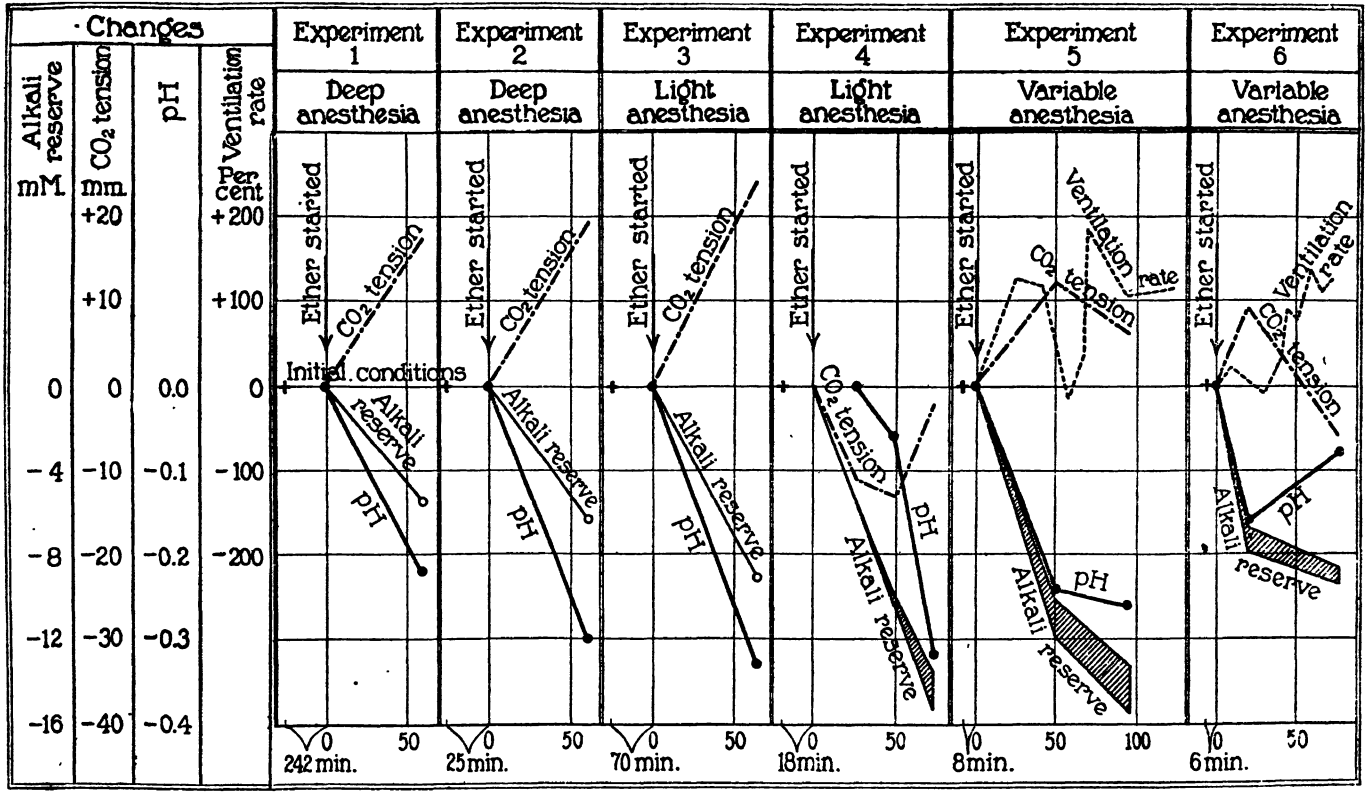
Changes
Alkali reserve
mM.
CO2 tension
mm.
pH
Ventilation rate
Per cent
+20
+10
0
0
0.0
0
+200
+100
−4
−10
−0.1
−100
−8
−20
−0.2
−200
−12
−30
−0.3
−16
−40
−0.4
Experiment 1
Deep anesthesia
Experiment 2
Deep anesthesia
Experiment 3
Light anesthesia
Experiment 4
Light anesthesia
Experiment 5
Variable anesthesia
Experiment 6
Variable anesthesia
Ether started
CO2 tension
Initial conditions
Alkali reserve
pH
Ventilation rate
0
50
100
242 min.
25 min.
70 min.
18 min.
8 min.
6 min.

FIG. 2.

determined from equilibration data, since data on each blood were determined only at one point, with CO_2 tension as it existed when the blood was drawn. We have accordingly constructed two graphs in each case, using the two extreme limits of the $\frac{d[BHCO_3]}{dpH}$ slope as determined in a number of dog bloods from experiments of our own and from experiments in the literature. These limits are $\frac{d[BHCO_3]}{dpH}$ $= -20$ to -28, $[BHCO_3]$ being expressed in millimol units. The two sets of alkali reserve changes estimated by extrapolation with these two slopes are indicated, for Experiments 4, 5, and 6, in Fig. 2 by two lines, with a shaded area between them. The width of this area indicates the maximum error introduced by this method of extrapolation.

In Fig. 2 we have plotted the results as changes in alkaline reserve, CO_2 tension, pH, and ventilation rate from the initial values determined before anesthesia.

In our tables we have given the absolute values of our data.

Experiments and Results.

The details of the six experiments are shown in the protocols and tables and the results are given graphically in Fig. 2. In Experiments 1 and 2 the anesthesia was as deep as possible. In Experiments 3 and 4 it was as light as possible, the animal making spontaneous movements from time to time. In Experiments 5 and 6 the animals were given an uneven anesthesia, being at times deeply under and at times almost out of ether but with the anesthesia becoming more evenly settled at about the middle of the second stage as the experiment progressed.

In Experiments 1 to 4 no special precaution was taken to prevent fall in body temperature and as indicated in the protocol of Experiment 4 some cooling occurred. In Experiments 5 and 6 fall in temperature was prevented by covering the animal with a blanket throughout the experiment. The results, however, were substantially the same.

Ventilation rate was studied only in the last two experiments. There is continual fluctuation in the ventilation rate but in both of

these experiments there is a tendency to its progressive increase in spite of the fact that in the latter part of the experiment the animals were held in the middle of the second stage of anesthesia.

SUMMARY.

In all the experiments it will be seen that the pH of the blood fell either at once or in a short time after the anesthesia was begun, and in the last three experiments it will be seen that although the pH remains low or continues to fall, the alkaline reserve also continues to fall. In no case was any rise of pH above the initial value observed. In all but one of the experiments the CO_2 tension was increased.

The evidence indicates that these changes do not occur as a compensatory mechanism to balance an acapnia. A true acidosis occurs with increase of the hydrogen ion concentration of the blood and fall of the alkaline reserve, due either to introduction of acid into the blood or to withdrawal of base from it.

Experiment 1.

Young male. 10.12 a.m. first bleeding, left ventricle; 2.15 p.m. ether started, drop method, deep anesthesia; 3.15 p.m. second bleeding, left ventricle.

Blood Equilibrated at 38° (Defibrinated).

Sample.	Time drawn.	CO_2 tension.	Total $[CO_2]$.	pH	
				Calculated.	Rectified by graph.
		mm.	*mM.*		
1	Before ether.	18.7	10.9	7.465	7.465
		37.4	15.2	7.300	7.315
		56.2	18.8	7.206	7.193
		74.9	20.9	7.118	7.118
2	After 60 minutes' deep etherization.	56.2	14.2	7.069	7.063*
		74.8	16.0	6.990	7.002*

* Graph drawn parallel to that of blood before ether.

Blood as Drawn.

Sample.	Time drawn.	Determined.			Calculated.				
		Total $[CO_2]$.	O_2 content.	O_2 capacity.	Total $[CO_2]$ oxygenated at same pH.*	pH by interpolation of CO_2.	CO_2 tension.	$[BHCO_3]$	
								As drawn.	At initial pH.
		mM.	*mM.*	*mM.*	*mM.*		*mm.*	*mM.*	*mM.*
1	Before ether.	16.4	9.70	10.15	16.2	7.27	42	14.9	14.9
2	After ether.	15.9	8.55	11.17	14.5	7.05	60	12.7	9.2†

* $\frac{d\,CO_2}{d\,O_2} = 0.52$ at constant pH (unpublished data).

† From extrapolated CO_2 (see Fig. 1).

Experiment 2.

Young male (same as Experiment 1). 9.25 a.m. first bleeding, left ventricle; 9.49 a.m. ether started, drop method, deep anesthesia; 10.51 a.m. second bleeding, left ventricle.

True Plasma from Oxalated Blood Equilibrated at 38°.

Sample.	Time drawn.	CO_2 tension.	Total $[CO_2]$.	pH	
				Calculated.	Rectified by graph.
		mm.	*mM.*		
1	Before ether.	40	18.2	7.224	7.224
		60	21.5	7.112	7.112
2	After 62 minutes deep ether.	20	9.8	7.257	7.257
		70	18.5	6.960	6.960

True Plasma (Oxalated) as Drawn.

Sample.	Time drawn.	Total $[CO_2]$.	Calculated.			
			pH by interpolation of $[CO_2]$.	CO_2 tension.	$[BHCO_3]$	
					As drawn.	At initial pH.
		mM.		*mm.*	*mM.*	*mM.*
1	Before ether.	18.2	7.22	40	16.9	16.9
2	After 62 minutes deep ether.	17.2	7.00	59	15.3	10.0*

* From interpolated $[CO_2]$.

Experiment 3.

Young male (same as Experiments 1 and 2). 10.45 a.m. first bleeding, left ventricle; 11.55 a.m. ether started, lightest possible anesthesia, drop method; 12.25 to 12.37 p.m. breathing very violently; 12.37 to 1.00 p.m. anesthesia very light, regular deep breathing, 33 per minute; 1.00 p.m. second bleeding, left ventricle.

Oxalated Blood Equilibrated at 38°.

Sample.	Time drawn.	CO_2 tension.	Total $[CO_2]$.	pH	
				Calculated.	Rectified by graph.
		mm.	*mM.*		
1	Before ether.	21.8	15.1	7.545	7.550
		50.8	21.0	7.305	7.230
		72.5	23.4	7.190	7.195
2	After 65 minutes light ether.	21.4	10.0	7.367	7.360
		50.8	15.6	7.165	7.180
		72.5	18.9	7.086	7.070

Blood as Drawn (Oxalated).

Sample.	Time drawn.	Total $[CO_2]$.	pH by interpolation.	Calculated.		
				CO_2 tension.	$[BHCO_3]$	
					As drawn.	At initial pH.
		mM.		*mm.*	*mM.*	*mM.*
1	Before ether.	18.6	7.40	41	17.7	17.7
2	After 65 minutes light ether.	18.9	7.07	75	17.1	8.3*

* From $[CO_2]$ interpolated at pH 7.40.

Experiment 4.

Young male. 1.10 p.m. first bleeding, left ventricle; 1.28 p.m. ether started, drop method, light anesthesia; 1.55 p.m. second bleeding, right femoral artery; 2.18 p.m. third bleeding, right femoral artery; 2.42 p.m. fourth bleeding, left femoral artery. Rectal temperature: initial and maximum 39.9; final and minimum 37.3.

True Serum as Drawn (Spontaneous Coagulation).

Sample.	Time drawn.	Determined.			Calculated.			
		$[BHCO_3]$ titration.	Total $[CO_2]$ gasometric.	Colorimetric pH.	CO_2 tension.	$[BHCO_3]$ as drawn from gasometric CO_2.	$[BHCO_3]$ at initial pH. $\frac{d[BHCO_3]}{d\,pH} = -20$	$[BHCO_3]$ at initial pH. $\frac{d[BHCO_3]}{d\,pH} = -28 \frac{dB}{d\,pH}$ observed
		mM.	*mM.*		*mm.*	*mM.*	*mM.*	*mM.*
1	Before ether.	21.5	23.0	7.29	54	21.7	21.6	21.6
2	After 27 minutes light ether.	12.8	16.8	7.29	40	15.9	16.0	16.0
3	After 50 minutes light ether.	12.8	13.8	7.23	37	12.9	11.7	11.2
4	After 74 minutes light ether.	12.6	13.5	7.07	51	12.3	6.3	6.3

Experiment 5.

Young male. 10.02 a.m. first bleeding, left ventricle; 10.10 a.m. ether started, drop method; 10.27 a.m. cannula in trachea, anesthesia kept in middle of second stage; 11.00 a.m. second bleeding, left femoral artery; 11.47 a.m. third bleeding, right femoral artery. Rectal temperature: initial 38.2°; final 38.3°; maximum 38.6°; minimum 38.2°C.

Sample.	Time drawn.	Total $[CO_2]$ oxalated blood as drawn.	Colorimetric pH. True serum as drawn.	Calculated for whole blood.			
				CO_2 tension.	$[BHCO_3]$ as drawn.	$[BHCO_3]$ at initial pH. $\frac{d[BHCO_3]}{d\,pH} = 20$	$\frac{d[BHCO_3]}{d\,pH} = 28$
		mM.		*mm.*		*mM.*	*mM.*
1	Before ether.	21.70	7.44	39	20.5	20.5	20.5
2	After 50 minutes varied ether.	16.78	7.20	47	15.4	10.6	8.7
3	After 97 minutes varied ether.	14.03	7.18	45	12.7	7.3	5.2

Time.	Ventilation rate.	Per cent Δ.
	liters per min.	
Day before.	5.6	
9.48 a.m.	3.9	
Mean before ether.	4.8	±0
10.35 a.m.	11.0	+129
10.52 "	10.4	+118
11.07 "	4.1	− 15
11.17 "	6.5	+ 35
11.20 "	13.6	+183
11.43 "	9.9	+106
12.14 p.m.	10.0	+108

Experiment 6.

About 4 year old male. 10.50 a.m. first bleeding, left ventricle; 10.56 a.m. ether started, drop method, kept about mid-second stage; 11.15 a.m. second bleeding, left femoral artery; 11.32 a.m. tracheal cannula introduced; 12.13 p.m. third bleeding, right femoral artery. Rectal temperature: initial 38.2°; final 38.3°; maximum 38.5°; minimum 38.2°C.

Separate Serum Equilibrated at 38°.

Sample.	Time drawn.	Atmospheric CO_2 tension.	Serum.			Calculated from colorimetric pH.			
			Total $[CO_2]$.	Electrometric pH 38°.	Colorimetric pH.	$[H_2CO_3]$	$[BHCO_3]$	$\log \frac{[BHCO_3]}{[H_2CO_3]}$	pK'
		mm.	*mM.*			*mM.*	*mM.*		
1	Before ether.	40.4	25.52	7.38	7.39	1.28	24.24	1.277	6.11
3	After 77 minutes varied ether.	39.4	18.65	7.19	7.19	1.25	17.40	1.144	6.05 Mean 6.08

True Serum as Drawn.

Sample.	Time drawn.	Total $[CO_2]$.	Colorimetric pH.	Calculated (using pK' = 6.08).			
				CO_2 tension.	$[BHCO_3]$ as drawn.	$[BHCO_3]$ at initial pH. $\frac{d[BHCO_3]}{d\,pH} = 20$	$[BHCO_3]$ at initial pH. $\frac{d[BHCO_3]}{d\,pH} = 28$
		mM.		*mm.*	*mM.*	*mM.*	*mM.*
1	Before ether.	25.32	7.36	40	24.06	24.1	24.1
2	After 19 minutes ether.	22.20	7.20	49	24.64	17.4	16.2
3	After 77 minutes.	18.16	7.28	34	17.08	15.5	14.8

Experiment 6—Concluded.

Time.	Ventilation rate.	Per cent Δ.
	liters per min.	
Day before.	4.4	
10.30 a.m.	4.6	
Mean before ether.	4.5	± 0
11.05 a.m.	5.6	+ 22
11.24 "	4.1	− 9
11.35 "	5.8	+ 29
11.41 "	8.4	+ 87
11.47 "	7.9	+ 76
11.55 "	10.6	+136
12.02 p.m.	9.6	+113
12.08 "	10.2	+127

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AN ANALYSIS OF CAMEL'S COLOSTRUM.

BY HELEN L. FALES.

(From the Laboratories of The Rockefeller Institute for Medical Research and the Babies' Hospital, New York.)

(Received for publication, June 8, 1922.)

Through the courtesy of Doctor W. T. Hornaday of the New York Zoological Society we were able to obtain for analysis a sample of the colostrum of a Bactrian camel, 2 days after parturition. Since reports of analyses of milks, other than those of the cow and goat, are none too plentiful it seems of possible value to have a published record of this analysis.

Colostrum from Bactrian Camel—2nd Day after Parturition.

Volume of sample	165 cc.
Appearance	Thick and rich, not yellow but creamy white.
Taste	Bland, less taste than cow's milk. Absolutely no unpleasant odor.
Reaction	Slightly amphoteric to litmus, acid reaction more marked.
Specific gravity	1.038
Fat	7.4 per cent
Sugar	4.2 " "
Protein	5.4 " "
Casein	4.1 " "
Albumin	0.5 " "
Globulins, etc.	0.8 " "
Ash	0.893
CaO	0.272
MgO	0.025
P_2O_5	0.318
K_2O	0.164
Na_2O	0.082
Cl	0.128

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THE TREATMENT OF RHEUMATIC FEVER.

BY HOMER F. SWIFT, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

The treatment of a sick patient depends largely upon the physician's conception of his disease. This is especially true if treatment consists not merely in alleviating pain or other unpleasant symptoms, but is directed towards increasing the patient's power to master the malady. It is well to recall that in all general infections there is a constant struggle between patient and infectious agent, and that recovery is usually brought about by an increase in the defensive mechanism of the host, while a downward course is evidence of a failure of these defensive agencies to keep pace with the inroads of the parasite. At times the struggle between these two antagonists is quickly terminated; the outcome is soon known; either recovery or death closes the picture. We witness an acute disease. Another degree of intensity gives us a chronic infection; the host has not sufficient strength completely to subdue the parasite, but the constant cohabitation of the two contestants is of such a nature that the struggle is prolonged: on the one hand, the various defensive agencies of the body are slowly mobilized so that the parasite is either killed or rendered harmless by encapsulation; on the other hand, conditions may be slightly in favor of the infectious agent and there follows either a continual destruction or an abnormal overproduction of certain tissues of the host. In other instances the clinical picture shows successive periods of advantage on one side and then on the other; the balance is often so fine that the slightest depressing influence upon the patient may permit the recrudescence of symptoms.

By suitable treatment the pathogenic properties of the virus may be kept at a level so low that ordinary diagnostic measures fail to detect its activity. It is entirely conceivable—even highly probable—that drugs or other parasitistatic agents may be sufficiently powerful to depress the virus to such a degree that it may be somnolent but not moribund; withdrawal of the drug may permit it again to resume

its nefarious work. It is possible, moreover, that a drug may prevent the virus from acting on one organ or set of organs and still be powerless to suppress its activity elsewhere. Thus the clinical picture of the disease may be so altered that it is difficult or impossible to recognize it as a condition analogous to that presented by an untreated patient.

The consideration of latency of infectious agents is one that would require much time to discuss fully. It is a most important phase of many diseases. Because of an increase of the patient's natural defenses the virus may be encapsulated, but remain living and capable of resuming its pathogenic activity should the immuring tissue be destroyed or other inhibitory agencies be depressed as the result of another disease. Again it is well recognized that a microörganism such as the Treponema pallidum may lie latent in tissues for long periods without exerting any demonstrable local destructive action, or inciting the usual cellular response; then, as a result of a lowering of the patient's general resistance, or because of a local trauma, lesions characteristic of syphilis may be produced. Similar examples might be cited in other infections. As one studies his patients over years he is more and more impressed with the probability that recurrences in many diseases are due to renewed activity of infectious agents that have lain latent in the tissues for months or years.

The various possibilities just mentioned must all be carefully weighed in considering rheumatic fever, its complications and sequelae. Unfortunately, we do not know the specific etiologic agent. Our classification of the various conditions included under the term, rheumatic fever, rest therefore upon a study of symptoms and signs, upon the peculiar course of these manifestations, upon the response of some of them to certain drugs, and, finally, upon characteristic histopathological alterations in certain organs. It is possible that our conception of the nature of this disease may be entirely altered by new discoveries; on the other hand, if we may draw an analogy from the history of the study of syphilis, it is probable that the discovery of a definite etiologic agent or the demonstration of characteristic immune reactions would extend the picture rather than alter entirely the notions already extant. Thus clinical study pursued with new methods as well as old will bring us nearer to a true picture of the relationship existing between the various manifestations in different cases.

In recent years an altered point of view towards this disease is reflected by the change in the name from acute inflammatory rheumatism, acute arthritis, or polyarthritis rheumatica, to rheumatic fever. It is evident that high fever, profuse perspiration, marked general intoxication and acute migratory polyarthritis responding to salicylates do not give us a complete picture of the disease; less obvious manifestations point to involvement of important viscera. For example, we now recognize that certain symptoms and signs, of themselves relatively insignificant, but important when considered in relationship to the general infection, indicate active disease in the heart. These are recurring fever and rapid pulse rate without renewed arthritis or evidence of complications in other organs; precordial pain and tenderness, and areas of paraesthesia in the so-called cardiac Head's zones; dyspnea and orthopnea in adults, and in children an increased respiratory rate unexplained by pneumonia or pleurisy; increased area of cardiac dulness; abnormal cardiac rhythms such as partial and complete heart block, auricular fibrillation, and tachycardia. Daily electrocardiographic studies of all of our rheumatic fever patients have convinced us that these symptoms and signs point to actual disease of the myocardium. Rarely is their instrumental evidence without accompanying clinical signs of cardiac abnormality. It is noteworthy that the majority of our patients minutely studied in the past three years show definite bedside and electrocardiographic evidence of myocardial involvement. If we add to this the long-known tendency for rheumatic fever patients to develop endocarditis and pericarditis, it is evident that most persons suffering from *polyarthritis rheumatica* also have *carditis rheumatica.*

In children and adolescents with rheumatic fever, arthritis is often so slight and transitory that it may be easily overlooked, and the entire picture be one of visceral involvement. Recently a child died in the Presbyterian Hospital with symptoms of acute heart failure. We were unable to obtain a history or other evidence of any of the usual rheumatic manifestations; microscopic examination revealed acute rheumatic myocarditis with many characteristic Aschoff bodies; no endocarditis or pericarditis. There is evidence, therefore, that the virus can attack the heart alone and allow all of

the other organs to escape injury. Many children with chorea have no fever, or one only of low grade, and show clinical evidence of active cardiac disease; post-mortem examinations reveal histopathological evidence of rheumatic myocarditis and endocarditis. Others may have, in addition, characteristic subcutaneous nodules, or there may be only nodules and carditis. Our present belief is that these various combinations of the rheumatic series (Cheadle) in childhood point to activity of the unknown virus in the body just as certainly as if there were a migratory polyarthritis. Other less distinct indications of probable continuing rheumatic infection during childhood are repeated attacks of tonsillitis, enlarged tonsils and satellite lymph nodes, growing pains, and certain cutaneous manifestations.

Failure to recover weight lost during the first weeks of illness or continuous loss of weight are presumptive evidence of persistence of infection. In recent years we have weighed all patients daily, and compared their weight charts with other signs and symptoms. Not only in children, but also in adults have we repeatedly observed that stationary weight below that usual for the patient is attended by slight fever, recurring arthritis or evidence of carditis; relapses have usually been heralded or accompanied by additional loss of weight or by a cessation of increase ordinarily evidenced by a recovering patient. A steady gain in weight does not necessarily mean that the patient will not suffer a relapse, but the liability is much less than in the presence of a subnormal, stationary, or falling weight. In this respect, rheumatic fever may be compared to tuberculosis. It has long been recognized that the acute stages with high fever and painful polyarthritis are attended by a rapidly developing anemia and emaciation. Clinicians who studied the disease before the introduction of salicylates describe the starved appearance of rheumatic fever patients who remained in the hospitals for months. Fortunately, the drugs at our disposal prevent most patients going into such a cachectic state; but the majority of them display a similar tendency which is masked unless we follow the weight curves. The loss of weight may be due to several causes: Du Bois and his co-workers have shown that fever *per se* increases the basal metabolism in a very definite manner. In severe infections the toxic state leads to a rapid destruction of body protein. A rough parallelism between

the level of basal metabolism and the pulse rate has been demonstrated by several observers. Frequently the pulse of patients with rheumatic fever remains rapid after the fever has disappeared. In many instances this persistently high pulse rate is evidence of a continuation of myocarditis, and suggests that the virus is still active. Thus we may regard a high pulse rate as an indication for rest, and also for increasing the patient's food intake.

Leucocytosis is another important sign of continued infection. It has long been known that leucocytosis was a feature of this disease, and that invasion of new joints or organs was accompanied by an increase in the number of white blood cells; there are numerous observations that the number of leucocytes falls after the exhibition of sufficient salicylate to eliminate the arthritis and fever. Lately we have been making leucocyte counts of all our patients several times a week, and have been impressed by the fact that after the initial fall in leucocytes accompanying the administration of salicylates or neocinchophen, there is often a recurring leucocytosis which persists in spite of continuing the drug in sufficient doses to keep the temperature normal and the patient free from pain. In order to test the meaning of this sign, patients have been treated in different ways and the result noted.

Our studies now indicate that leucocytosis is an evidence of a persistent infection, and also confirm the opinion derived from other observations that the salicylates and neocinchophen do not completely destroy the virus, but suppress it sufficiently to relieve many of the characteristic symptoms of the disease.

This leads us directly to a consideration of salicylic acid with its numerous derivatives; later, compounds of phenylcinchoninic acid will be discussed.

From the clinical viewpoint probably no more striking therapeutic effect has been seen than that following the introduction of the salicylates for the treatment of acute articular rheumatism. The results were marvelous; intense suffering was relieved, and the distressing sequelae incident to continued high fever and prolonged intoxication were eliminated. The failure to elicit a similar response in patients with other conditions led to the opinion that salicylic acid was a specific for acute inflammatory rheumatism. The activity of syn-

thetic chemists has resulted in numerous derivatives; drugs less irritating to the gastro-intestinal tract than the crude salicylic acid or sodium salicylate, but all dependent upon the salicyl ion for their specific action. Pharmacologists have shown that in common with many coal tar compounds, the salicylates have an antipyretic action due to increased heat elimination. They also are analgesics, they lessen pain in many conditions, both non-rheumatic and rheumatic. But the disappearance of the signs of the inflammation,—swelling, heat, redness, and tenderness,—so strikingly evident in the joints of rheumatic fever patients following the exhibition of large doses of salicylates, is not seen in patients with arthritis of known bacterial origin, nor in animals with experimental arthritis. The so-called specific action of the drug is, therefore, unexplained. This is not surprising when we realize that the etiology of rheumatic fever and the exact nature of the arthritis are unknown. Certain observers have claimed that the antiphlogistic effects of salicylates demonstrated in the arthritis of patients with acute rheumatic fever are not specific. By means of special charts, whereon all of the symptoms and signs of inflammation in each joint are noted daily, we have recorded in many cases the results following the administration of therapeutic doses of salicylates; many of the patients have been observed for varying periods before administering the drug; the evolution and resolution of the inflammation in each single joint and in all of the joints have been followed; repeatedly we have recorded the sudden disappearance of inflammation in recently invaded joints and as striking a cessation of the tendency for new joints to be involved. At the same time we have failed to observe a similar antiphlogistic effect in patients with gonorrheal rheumatism, or arthritis due to other bacteria; patients with serum disease arthritis, likewise, have not been relieved by large amounts of salicylates. These observations are not unique, nevertheless they are of distinct value because made with a definite point of view: to prove the specific antiphlogistic action of salicylates upon the arthritis of rheumatic fever patients.

The therapeutic and toxic doses of salicylates approximate one another. Failure to recognize this fact often results in failure to allay pain. Frequently we have admitted patients who have been taking 0.6 to 1.0 gram (10 to 15 grains) doses of sodium salicylates

or aspirin three or four times daily with only partial or no alleviation of their symptoms; subsequent rapid administration of the same drug to a point just below toxicity has afforded complete relief.

The physician should be thoroughly familiar with the symptoms and signs of toxicity: tinnitus aureum, deafness, nausea, vomiting, flashes of light; in extreme instances, delirium; injury to the kidney resulting in hematuria and diminished renal function are occasionally seen if the drug is given too freely. Hanzlik and his co-workers have laid special emphasis upon renal poisoning, and claimed that many of their patients had a temporary gain in weight after receiving amounts of sodium salicylate sufficient to relieve their arthritis and fever. We have repeated these experiments but failed to observe such marked evidence of renal injury, either in the form of severe albuminuria, cylindruria, hematuria, or evidence of an accumulation of edematous fluid in the patient's subcutaneous tissue. Gain in weight in practically all instances could be explained upon the basis of replacement of water previously lost by excessive diaphoresis. Rarely, when salicylates have been given in large amounts, we have seen edema of the extremities and a sudden increase in weight; but in such cases it was subsequently evident that too much had been administered. Our rule is to prescribe the drugs in 1 to 1.5 gram (15 to 22 grains) doses every hour until pain is alleviated or the first toxic symptoms appear—usually tinnitus or nausea; ordinarily from 6 to 12 grams (90 to 180 grains) are sufficient. The drug is then discontinued until the following day when a total amount of from one-half to three-fourths of the toxic dose is ordered. Depending upon the severity of the case and the therapeutic results the drug is continued in this quantity for longer or shorter periods, and subsequently the daily total is slowly reduced. We have found that an hourly chart on which are noted the therapeutic and toxic effects of the drug for the first two days, and a daily chart on which the joint symptoms, temperature, and pulse rate are recorded have been of great value in helping us determine the correct dosage for each patient.

Individualizing in the administration of salicylates to rheumatic fever patients is most important. No absolute rule can be given except that of studying each patient during each course of treatment. We have seen the toxic dose vary between 6 and 10 grams for the

same individual at different times. An even wider range is seen in different individuals. Men, as a rule, tolerate larger doses than women. The body weight of the patient probably bears some relation to the toxic and therapeutic dose.

The beneficial effect of practically all of the numerous derivatives of salicylic acid is more or less proportional to the salicyl ion content. Improvements in forms of the drug concern themselves mainly with compounds that are less irritating to the gastro-intestinal tract. Crude salicylic acid is most irritating and practically never given; sodium salicylate is less irritating but often is repulsive because of its peculiar taste. Acetylsalicylic acid having a sour taste is often better tolerated. The list of salicyl derivatives is too long to describe in detail, and is probably much larger than necessary. Numerous preparations have been introduced to the profession by enterprising drug manufacturers with glowing testimonials. Had physicians been well acquainted with the technique of using sodium salicylate and acetylsalicylic acid properly they would rarely have been compelled to resort to more unusual and expensive derivatives of salicylic acid in the treatment of patients suffering from rheumatic fever. There is no doubt that certain patients can tolerate therapeutic doses of acetylsalicylic acid when they cannot bear sodium salicylate; the reverse is also true. Cases are also seen where other derivatives appear to be better tolerated; but often it will be found that the apparent diminution in toxicity is due to a lower salicyl content of the preparation in question; and the therapeutic effect is proportionally less.

Attention has already been drawn to certain actions of the salicylates. It is important to realize that we do not know the real reason for the so-called specific effect, although there has been much speculation and some experimentation on this subject. Hanzlik and his co-workers have shown that the salicyl ion does not exist in any greater concentration in the joint fluid than in other tissues, and also that it probably does not exist as free salicylic acid in arthritic exudates. Boots and Cullen have found that the reaction of joint exudates from patients with rheumatic fever is always alkaline, so that free salicylic acid cannot exist in these exudates even though large amounts of salicylic salts were present. The anti-arthritic effect cannot, therefore, be due to free salicylic acid.

Some experiments of mine indicate that during immunization animals receiving sodium salicylate in doses comparable to the largest therapeutic doses for patients produce somewhat smaller amounts of immune bodies than do controls similarly immunized but not receiving salicylates. This suggests that possibly this drug acts by depressing the susceptibility of the animal to the antigenic stimulus of the foreign protein—both bacterial and non-bacterial. In another series of experiments by Boots and myself, salicyl treated rabbits with experimental arthritis induced by intravenous inoculation of green streptococci had fewer purulent joints than did a corresponding number of non-salicylated controls. Both groups of animals had a similar number of inflamed joints; the smaller amount of purulent arthritis among the salicyl treated animals suggests that in some instances, at least, the drug decreased the irritating power of the bacteria in joints even though the joints were infected. This suggests that the action of salicyl in the animal body may be bacteriastatic even if it is not actually bactericidal.

Such an action corresponds with the observed effect in patients with rheumatic fever. The persistent leucocytosis, the continuation of signs of myocarditis, the development of endocarditis and pericarditis in patients under the influence of salicylates, and the liability for many patients to have relapses when the drug is reduced too rapidly or discontinued too soon, all indicate that its chief action is to reduce the intensity of the pathologic process rather than to destroy completely the virus. It seems to us that this reduction in intensity of the disease relieves pain, spares the patient, and permits a more rapid convalescence.

Such effects might lead to harmful therapy if not well understood and considered in the general management of the patient. Relieving certain distressing symptoms and lessening others so that the patient feels almost well may lull both the sick man and physician into a false sense of security. Very frequently I have seen patients who, while taking 2 to 5 grams (30 to 75 grains) of sodium salicylate or aspirin daily have been discharged from a hospital, and after a few days at home without the drug have developed relapses of varying intensity. According to our present opinion such patients were suffering the entire time from the disease, but were unconscious of

its ravages; they were, therefore, subjecting themselves to stress and strain beyond a proper point, and losing the benefit of the time already spent in the hospital, and increasing their liability to serious visceral complications. Under such circumstances it is questionable whether the administration of drugs may not do more harm than good. This statement is not an indictment of anti-rheumatic remedies but rather a common method of using them.

Recently phenylcinchoninic acid and its derivatives have been introduced for the treatment of rheumatism.* Derivatives of quinolene, they were first used for the treatment of gout. Although unrelated, both salicylic acid and phenylcinchoninic acid have been found to increase the permeability of the kidneys for several non-protein nitrogen waste products; both are antipyretics, and both decrease the signs of inflammation in the joints of rheumatic fever patients. We have found that cinchophen and neocinchophen act against the signs and symptoms of rheumatic fever in much the same manner as do the salicylates. Cinchophen is liable to induce severe gastric distress in patients taking enough to relieve their arthritis and pyrexia. Neocinchophen, on the other hand, has been free from this unpleasant by-effect, and has been given in daily total doses of from 0.1 to 0.15 of a gram per kilo body weight without severe demonstrable toxicity. All of the remarks already made in reference to the therapeutic action of the salicylates can be applied to drugs of the cinchophen series: they combat certain unpleasant symptoms, but clinically do not seem to destroy the virus completely, as many patients have continued to have a leucocytosis while under their influence and others have developed relapses upon discontinuing the drug. Similar to our experience with both sodium salicylate and aspirin, we have occasionally seen patients continue to develop new symptoms and signs while taking therapeutic quantities of neocinchophen, and experience marked relief when another anti-rheumatic remedy was substituted. Neocinchophen is a useful adjuvant to our therapeutic armamentarium, but up to the present has not been proven a substitute for the salicylates. Each drug at times is useful in replacing the other, when the

* Atophan was the name first used, but during the war the term cinchophen was adopted. Similarly, neoatophan was changed to neocinchophen. Another trade name is tolysin.

one first tried is either too toxic or fails to induce the proper therapeutic effect.

No satisfactory serum has been devised for the treatment of patients with acute rheumatic fever. This is not surprising in view of our ignorance of an etiologic agent with which to immunize animals. It now seems probable that the favorable results from anti-streptococcus serum injections reported several years ago were similar to the recoveries seen following the injection of other forms of foreign protein.

Patients with all types of arthritis have been treated by intravenous or intramuscular injection of killed bacteria, various colloidal substances, or other foreign proteins in various forms. Typhoid vaccine seems to have been employed more than other foreign proteins. In order to obtain satisfactory clinical results it is necessary to induce a severe chill, followed by fever, symptoms of marked general intoxication and to stimulate a high degree of leucocytosis. Similar treatment has been employed in most general and local infections as well as in the treatment of arthritis. It is now recognized that there is nothing specific in this form of therapy; that when improvement follows it appears to be due to a sudden mobilization of leucocytes, and possibly other defensive agencies. Many observers who claim the most favorable results from intravenous injections of bacterial vaccines into rheumatic fever patients report only an improvement or disappearance of the arthritis, and an antipyretic effect; they do not mention whether the other symptoms of the disease disappear or not. All agree that the toxic reaction is too severe to warrant the general introduction of this form of therapy, and advise that it be reserved for those patients who do not respond favorably to the salicylates. We have not attempted to repeat the observations of others because we felt that because of the non-specific nature of the reaction it would not help greatly in the elucidation of our problem of the nature of rheumatic fever, especially when such marked benefit can be obtained from drugs.

No discussion of treatment would be complete without mentioning the question of tonsillectomy or the removal of other foci of infection. There can be little doubt that there is a relationship between tonsillitis and rheumatic fever; the exact nature of that relationship is

another question, and one we shall not take up at this time. The proper removal of diseased tonsils is often followed by marked improvement in patients who are suffering from chronic forms of this disease. Even from the most conservative point of view, in which it might be claimed that tonsillitis is not "rheumatic" in nature, it would seem advisable to remove simple diseased tonsils, or tonsils that were the point of origin of other general infections. Even more, then, would one advise tonsillectomy if he believes that diseased tonsils are directly responsible for an attack of rheumatism. It is, however, well to bear in mind that many relapses occur in individuals who have had their tonsils well removed, and to warn patients of this possibility in advising operation.

SUMMARY.

Within the limits of this paper it has been impossible to consider the treatment of the various important complications of rheumatic fever, or to give specific directions for the use of the remedies discussed. An attempt has been made rather to summarize our present conception of the disease as an infection lasting longer than is ordinarily believed, to show that only certain of the well-recognized symptoms yield readily to anti-rheumatic drugs, and that these drugs often simply depress or mask the activity of the virus. In general, it is important to recognize what our remedies fail to do as well as what they do. Proper treatment consists not only in administering anti-rheumatic drugs, injecting foreign protein, or removing possible points of focal infection, but also in prescribing rest, guarding the heart against over-strain, regulating exercise, attending to the diet, and in devising measures to increase the patient's general resistance against infection.

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THE QUANTITATIVE INFLUENCES OF CERTAIN FACTORS INVOLVED IN THE PRODUCTION OF CYANOSIS.

BY CHRISTEN LUNDSGAARD AND DONALD D. VAN SLYKE.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, July 7, 1922.)

Lundsgaard has shown that the appearance of cyanosis depends on the mean concentration of reduced hemoglobin (C) in the capillary blood. This concentration he estimated as

$$C = \frac{A + V}{2} \tag{1}$$

where A is the arterial, V the venous concentration of reduced hemoglobin. The effect of certain physiological factors contributing to C is estimated as follows. We let T represent the total hemoglobin concentration in the blood, l the fraction of total hemoglobin passing in reduced form through the aerated parts of the lungs, D the concentration of reduced hemoglobin formed by deoxygenation of the blood as it passes from arteries to veins through the tissue capillaries. In certain pathological conditions a fraction of the venous blood reaches the arteries without traversing aerated parts of the lungs and therefore carries reduced venous hemoglobin directly into the arterial blood. This fraction we designate as α. A, T, and C have the significance indicated above.

When $\alpha = O$, only aerated blood entering the arteries, $A = l\,T$. When, however, α has a positive value, the reduced hemoglobin of the arterial blood represents the sum of that in the fraction α, of venous blood entering the arteries, plus that of the fraction $(1 - \alpha)$ of arterial blood from the lungs. In such a case

$$A = (1 - \alpha)\,l\,T + \alpha V \tag{2}$$

$$\alpha = \frac{A - l\,T}{V - l\,T} \tag{3}$$

Substituting $V - D$ for A in (3) and solving for V,

$$V = l\,T + \frac{D}{l - \alpha} \tag{4}$$

Substituting $A + D$ for V in (3) and solving for A,

$$A = l\,T + \frac{\alpha D}{1 - \alpha} \tag{5}$$

We now substitute for A and V in (1) their values as expressed in (4) and (5); and obtain

$$C = l\,T + \frac{(1 + \alpha)D}{2(1 - \alpha)} \tag{6}$$

which indicates the effects of the four factors, l, T, α, and D on C.

An interesting point brought out by (6), and in accord with observation, is that other factors normal about 40 per cent of venous blood may be mixed with arterial ($\alpha = 0.4$) before the reduced hemoglobin concentration in the latter becomes great enough to cause cyanosis.

The effects on C of separate variations of l, T, D, and α are indicated by partial differentiation of (6).

$$\partial C = T\,\partial l \tag{7}$$

$$\partial C = l\,\partial T \tag{8}$$

$$\partial C = \frac{D}{(1 - \alpha)^2}\,\partial\alpha \tag{9}$$

$$\partial C = \frac{(1 + \alpha)}{2(1 - \alpha)}\,\partial D \tag{10}$$

While the reduced hemoglobin concentration in the capillary blood may be accepted as the cause of cyanosis, there are various other factors which modify the resulting coloration. Such are local skin vascularity, pigmentation, thickness of epidermis; and also the fact that the mean capillary content of reduced hemoglobin $\frac{1}{2}(A + V)$ only approximately represents the average content. With changing conditions the latter may instead of being midway between venous and arterial, approximate either more nearly than the other. The effect of these modifying factors is to cause the value of C at which cyanosis becomes perceptible to vary from 4 to 6 gms. of reduced hemoglobin per 100 cc. of blood, and perhaps sometimes even more widely, although it appears usually to lie near 5.

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STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

I. TECHNIQUE FOR COLLECTION AND ANALYSIS OF BLOOD, AND FOR ITS SATURATION WITH GAS MIXTURES OF KNOWN COMPOSITION.

BY J. H. AUSTIN, G. E. CULLEN, A. B. HASTINGS, F. C. McLEAN, J. P. PETERS, AND D. D. VAN SLYKE.

(From the Hospital of The Rockefeller Institute for Medical Research.)

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INTRODUCTION.

Recent articles by L. J. Henderson (1920, 1921), Parsons (1919), Van Slyke (1921, *b;* 1922), and A. V. Hill (1922) have summarized the present state of our knowledge concerning the interaction of oxygen, carbon dioxide, hemoglobin, chlorides, and other constituents involved in the respiratory function of the blood and in the maintenance of its neutrality and osmotic pressure. The field in its present state has been developed from the blood gas studies of Bohr, Haldane, Barcroft, and their collaborators, and the investigations of the blood electrolytes dating from Zuntz through Hamburger and Gürber to present investigators (*e.g.* L. J. Henderson, 1908; Van Slyke and Cullen, 1917; Adolph and Ferry, 1921; Fridericia, 1920; Doisy, Eaton, and Chouke, 1922; Barcroft, Bock, Hill, Parsons, Parsons, and Shoji, 1922).

The reactions known to be involved in the respiratory changes of the blood, and the accompanying shifts of gases and acids between plasma and cells, are in part indicated qualitatively by the accompanying diagram (Fig. 1).

All six reactions are forced from left to right by increase in H_2CO_3, which results in formation in the plasma of bicarbonate from two sources (Reactions 1 and 2), and in the cells from two other sources (Reactions 5 and 6).

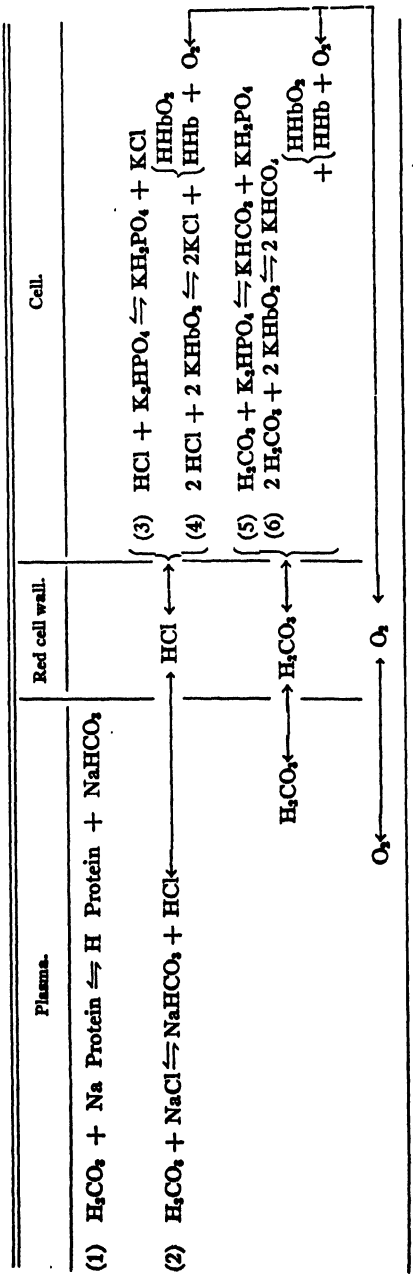
Plasma.
Red cell wall.
Cell.
(1) H_2CO_3 + Na Protein ⇌ H Protein + $NaHCO_3$
(2) H_2CO_3 + NaCl ⇌ $NaHCO_3$ + HCl
HCl
(3) HCl + K_2HPO_4 ⇌ KH_2PO_4 + KCl
(4) 2 HCl + 2 $KHbO_2$ ⇌ 2KCl + HHbO / HHb + O_2
(5) H_2CO_3 + K_2HPO_4 ⇌ $KHCO_3$ + KH_2PO_4
(6) 2 H_2CO_3 + 2 $KHbO_2$ ⇌ 2 $KHCO_3$ + HHbO / HHb + O_2
H_2CO_3
H_2CO_3
O_2
O_2

FIG. 1.

The displacement of base from combination with hemoglobin by HCl (Reaction 4) or H_2CO_3 (Reaction 6) results in a decrease in the oxygen bound by hemoglobin (last steps of Reactions 4 and 6), because when alkali hemoglobinate loses part of its alkali it also loses part of its affinity for oxygen.

On the other hand, increase of O_2 (from the lungs) forces Reactions 4 and 6 backwards, and thereby through Reactions 6 and 2 sets H_2CO_3 free.

By the reactions outlined influx of oxygen sets H_2CO_3 free and thereby helps the blood throw off in the lungs its overload of CO_2; while in the capillaries influx of CO_2 sets oxygen free from combination and thereby makes it more readily available to the tissues.

It is apparent from the diagram that every reaction affects every other reaction. In a given blood the O_2 and the H_2CO_3 concentrations are the two variables that are directly changed by respiration, and their alterations govern the accompanying changes in all the other variables, which are dependent on them. As L. J. Henderson has pointed out (1921), when we have in a given blood only two independent variables to deal with, by determining the relationship of each other variable to these two, or to any given two within the system, the quantitative interrelationships among them all may be determined. This may be accomplished either algebraically, or, more simply, by a two dimensional diagram such as Henderson has used (1921), or by an alignment chart. (The pH, although not indicated on our diagram, is also one of these dependent variables, since its value is set by the $BHCO_3$ and H_2CO_3 according to Hasselbalch's (1917) equation $pH = pK' + \log \left(\frac{[BHCO_3]}{[H_2CO_3]} \right)$.

It appears that the chief substances and reactions involved in the respiratory changes of the blood are probably known; and that Henderson has solved the mathematical problem of expressing by a practicable method the many relationships involved.

One cannot, however, read the recent theoretical papers quoted at the beginning of this paper without being struck by the present inadequacy of experimental data sufficiently accurate and complete to permit within definable limits of error the formulation of the

quantitative relationships involved.[1] Such data require simultaneous observations of several changes with technique of a precision gauged in detail to meet the requirements of the problem.

Preliminary experiments leading towards the systematic determination of data on the various factors in the system were begun by McLean, Murray, and L. J. Henderson (1920) in Henderson's laboratory. The magnitude of the task made a coordinated division of it between at least two laboratories advisable, and the present series of papers from this laboratory is the partial result of such a division.

The problem of the determination of CO_2 and O_2 tensions by analyses of the gas phase was already solved by Haldane's apparatus for air analysis. We used this apparatus with the single 4-way stop-cock introduced by Yandell Henderson (1918).

The methods available for the determination of blood gases were less satisfactory. Fig. 2 is constructed from observations collected from the literature by Peters, Barr, and Rule (1920), and presents in graphic form the relations which appear to obtain between the carbon dioxide tension, the carbon dioxide concentration, and the hydrion concentration in average normal human blood. On this figure have been introduced carbon dioxide absorption curves (a), of completely oxygenated blood; (b), of completely reduced blood (located at the distance above the curve of oxygenated blood indicated by the results of Christiansen, Douglas, and Haldane (1914)); and (c), of plasma from the oxygenated blood. A comparison of these curves shows the magnitude of the changes which it is necessary to analyze.

The large unblocked rectangle (A) represents an estimation of the maximum combined error involved in the determination of the carbon dioxide absorption curve by a technique employed recently by Peters, Barr, and Rule (1920). The error is indicated in terms of pH, of volumes per cent of carbon dioxide in the blood, and of millimeters of carbon dioxide tension. We believe that the procedure employed in these experiments was as accurate as that used by previous workers. Nevertheless, the rectangle representing the possible cumulative error

[1] The quantitative relationships between Reactions 1 and 2 appear satisfactorily established in a paper by Doisy, Eaton, and Chouke (1922), which appears as this paper goes to press.

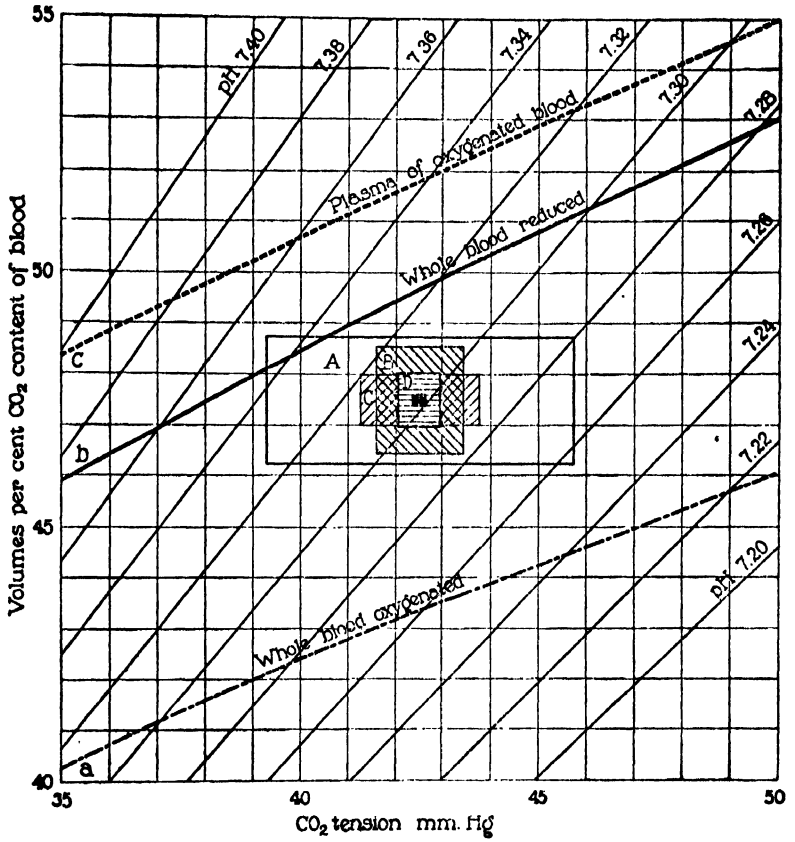

FIG. 2.

A = Maximum combined error estimated to exist in method used by Peters and Barr for the determination of the CO_2 absorption curve. (2.5 vol. per cent)

B = Error ascribed by Hasselbalch to the electrometric method of determining pH. (0.02)

C = Maximum error of earlier methods of measuring the carbon dioxide content of blood. (1.0 vol. per cent)

D = Error of present electrometric pH measurements. (0.01)

E = Small black square in center represents variations in duplicate determinations of CO_2 content by improved method now employed. ($\pm$0.1 vol. per cent)

covers 40 per cent of the difference between the carbon dioxide content of completely reduced and that of completely oxygenated blood. The cross-hatched rectangle (C) represents the limits of variation in duplicate determinations of the blood carbon dioxide by the method presented by Van Slyke in 1917 which was used by Peters, Barr, and Rule. These limits are of the same magnitude as those usually ascribed to the Barcroft and Haldane methods. The difference between the clear rectangle (A) and the cross-hatched rectangle (C) indicates the sum of the errors introduced by the manipulations which preceded the analyses. Although these errors are only estimated, we believe the estimates are approximately correct.

By improvements in apparatus and procedure for blood gas determinations (Van Slyke and Stadie, 1921), it is now possible to obtain consistently duplicate carbon dioxide readings with an average variation from the mean of ± 0.1 volume per cent or ± 0.05 millimol, a value represented by the small solid black square. A more recent improvement[2] has reduced the *maximum* variation in results by a skilled analyst, to ± 0.05 millimol of either CO_2 or oxygen. This variation is of about the same magnitude as that entailed in the volumetric measurement of blood in a pipette calibrated to deliver 1 cc. of water.

Variations in chloride estimations may be reduced to ± 0.1 millimol by the application of the method of Austin and Van Slyke (1920, 1921) to sufficiently large samples of blood.

The analytical errors having thus been reduced, it remained to develop a procedure for preliminary treatment which could be reproduced so accurately that two specimens of blood subjected to it would not differ in composition from each other, or from their common curves, by more than the analytical errors. It is the purpose of this paper to present a technique for the preliminary treatment of blood which will meet these requirements.

Table I presents the steps in the procedure of an ordinary experiment, and the factors which are active in the production of error.

[2] The principle of this apparatus was published last year in a preliminary note (Van Slyke, 1921, *a*). The details will appear shortly by Van Slyke and Neill in this *Journal*.

TABLE I.

Factors Involved in Determining Gas and Electrolyte Equilibria in Blood.

Steps in procedure.	Sources of error.
The drawing, preparation, and preservation of blood..... 1	Hemolysis.
The saturation of blood at a desired temperature with a desired gas mixture..... 2	Formation of non-volatile (lactic?) acid.
Determination of the exact composition of the gas mixture at equilibrium..... 3	Change of equilibrium conditions during separation of gas and liquid phases.
The delivery of blood from the tonometer into a receiving vessel..... 4	Change of gas content of blood by exposure to air or oil.
Separation of serum or plasma..... 5	
Preservation of blood, plasma, or serum for analysis..... 6	Change of gas content by exposure. Formation of non-volatile acid (whole blood only). Formation of CO_2 and consumption of O_2 (whole blood only).
Analyses of blood, plasma, or serum.... 7	Change of gas content by exposure during transfer of sample from container to apparatus for analysis. Uneven mixture of cells in whole blood at moment of measuring sample. Limit of accuracy of methods for analysis of gas and liquid phases.

Sources of Error.

1. Hemolysis.—Hemolysis changes the distribution of gases and electrolytes between cells and plasma. We avoided hemolysis by careful handling, and by using for most of our animal experiments horse blood, the cells of which are less fragile than those of dog blood. In the case of dog blood, we generally used serum in place of plasma, when determinations on the cell-free fluid were required.

2. Formation of Non-Volatile Acid in Blood.—Christiansen, Douglas, and Haldane (1914) showed that if defibrinated human whole blood is kept at 37° its carbon dioxide capacity falls by as much as 2 volumes

per cent in a half hour, apparently because of the formation of non-volatile acid. Peters, Barr, and Rule (1920) observed a similar acid formation in human blood, both defibrinated and oxalated, but found that when the blood was allowed to stand at room temperature after being drawn it showed no significant changes during the 1st hour.

The formation of acid is confined to the cells, since it does not occur in separated plasma. The time of onset varies in bloods of different species. Dog blood has been observed to change measurably in an hour at room temperature, while horse blood has shown no measurable change in several hours. In a paper which appears as this goes to press, Evans (1922) suggests that the non-volatile acid is formed by glucolysis, since its rate of formation parallels that of glucose disappearance. He finds that the acid formation is accelerated by loss of CO_2 (increase in pH) and is retarded by the addition of 0.1 per cent of sodium fluoride.

Up to the present we have avoided error from acid formation by working with horse blood, and by chilling the blood to zero whenever any time was allowed to elapse between the withdrawal of the blood and its exposure to a gas mixture or between this exposure or centrifugalization and analysis. We have also used uniform periods for saturation at 38°, so that if acid formation should occur it would be relatively constant in different blood samples. As a control of this factor, in experiments which involved a long series of exposures of samples of a given blood, we have repeated the conditions of the first exposure on the last of the series. Even when the chilled horse blood was permitted to stand some hours between the first and last exposures, no change was noted.

3. Formation of CO_2 and Consumption of O_2 by Metabolism of Whole Blood.—It has been shown by Harrop (1919) that, by this process, oxalated normal human blood loses 0.1 to 0.4 volumes per cent of oxygen in 6 hours at 38°. In horse blood we have found no significant changes at room temperature in an hour, which was the maximum time that blood was allowed to stand unchilled between withdrawal or saturation and analysis.

4. Uniform Mixtures of Cells and Plasma.—The necessity of obtaining a uniform mixture before samples are taken for either analy-

sis or saturation is especially great in horse blood, because the cells settle with unusual rapidity. The uniformity of mixture attained by our procedures was controlled by oxygen capacity determinations, and the procedures were regulated accordingly. The tubes in which

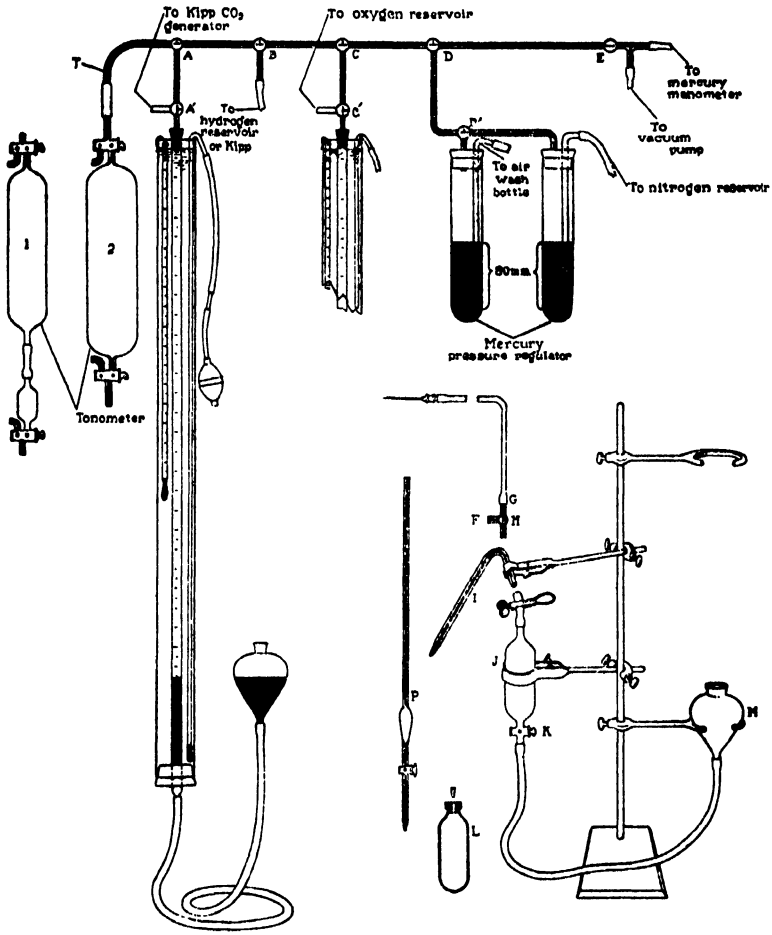

FIG. 3.

blood was kept over mercury (Tube *J*, Fig. 3), for example, were inverted twenty times immediately before a sample was withdrawn.

5. Change of Equilibrium Condition During Separation of Gas and Liquid Phases.—Changes in temperature or pressure, such as may result when the tonometer is removed from the bath, may so disturb

conditions of gaseous equilibrium existing in the tonometer as to render it impossible to obtain results sufficiently accurate for the purposes of the problem outlined above.

In the procedure we have usually used ("First saturation method" outlined below), the gas and liquid phases, in a state convenient for analysis, have been separated while the tonometer was still in the water bath by a mechanical device which avoided changes of temperature and pressure. In a second process, used for large amounts (over 30 cc.) of blood, the initial composition of the gas phase was fixed with especial care, and the amounts of CO_2 and O_2 taken from or given to it by the blood during saturation were calculated from accurate analyses of the blood before and after the saturation. Consequently analysis of the gas phases was avoided.

6. Collection and Preparation of Blood. (*a*). *For Saturation without Previous Analysis.*—When blood is to be exposed to known gas mixtures prior to all analyses, rigid precautions to avoid changes in gas content before exposure are, of course, not required. We have, however, avoided gross losses of carbon dioxide, such as might possibly cause irreversible changes, by drawing the blood under oil in cylinders arranged as described by Van Slyke and Cullen (1917, Fig. 1).

When oxalated blood was desired, potassium oxalate of tested neutrality was placed in the receiver in the proportion of 0.3 gm. to 100 cc. of blood. A saturated solution of neutral potassium oxalate was spread in a film on the walls of the receiver and dried by an air current. The neutrality of the oxalate was tested by the addition of phenol red to a diluted sample. Some samples of oxalate are alkaline. To such, oxalic acid was added until the pH was 7.4.

When defibrinated blood was required the oxalate was omitted, and the blood was defibrinated by stirring under oil with a rod. The blood was then filtered under oil through gauze.

(*b*). *Collection and Centrifugation of Blood for Analysis.*—When it was necessary to ascertain the gas content of the blood as drawn, as well as after exposure to a known gas mixture, we either drew it under oil by the above procedure or used the apparatus shown in Fig. 3. When small samples (10 to 20 cc.) were drawn under oil, the precaution was taken to use rubber and glass tubes (the latter

Pyrex) of only 2 mm. diameter for the blood to pass through, so that it would form a solid column without bubbles.

The receiving vessel, *J*, coated inside with oxalate, is connected with the mercury bulb *M* and the 3-way cock *H* bearing the needle. *J* and its connections up to *H* are filled with mercury, a few drops of which are wasted through *F*. The needle is then inserted into the blood vessel, and a few drops of blood are permitted to escape from F, in order to abolish the air-space remaining in the connecting tube. By turning *H* (with *M* in the lower position shown in Fig. 3) the needle and collecting vessel are directly connected and the blood is drawn into the latter. We have used tubes of the type *J* of from 10 to 200 cc. capacity.

As soon as the blood has been drawn *J* is inverted a number of times in order to insure mixing of the blood and oxalate, a process which is assisted by the mercury that remains. The leveling bulb *M* is kept at the upper level, in order to keep positive pressure on the blood sample.

7. Sampling Blood.—The cells and plasma are thoroughly mixed immediately before sampling by repeatedly inverting *J*. The leveling bulb being in the upper ring, the pipette *P* is inserted into the rubber tube outlet of *J*, the pinch-cock is opened, *K* is opened, and blood is allowed to flow up into the pipette.

8. Centrifugation without Loss of CO_2.—We have used centrifuge tubes of the type *L* in Fig. 3, similar to that described by Parsons (1919). A Pyrex centrifuge tube is fitted with a 1-hole stopper with a flange. Mineral oil is placed in the open tube, and the blood is allowed to flow under the oil and displace all but the last drops of oil from the tube. The stopper is then inserted, forcing out the remaining oil through the hole in the rubber. The glass plug is inserted in the hole, and the tube is ready for centrifugation.

After centrifugation the glass plug is removed, and a pipette containing oil is inserted in the hole. When the stopper is removed the oil flows over the surface of the plasma and prevents the escape of CO_2 during transfer of the plasma to the sampling tube *J*. For the protection needed, mineral oil is adequate, since it is in contact with the blood only a short time during which the blood is not agitated.

As an alternative to stoppering the centrifuge tube as above described, the surface of the blood may be covered, after most of the mineral oil has been removed, by a layer of melted paraffin (melting

point 40–45°) which need not exceed 5 mm. in thickness. After centrifugation, a little mineral oil is poured over the paraffin. A transfer tube or pipette may then be inserted through or along side the paraffin to remove the plasma. This technique is useful if the amount of blood is too small to fill the centrifuge tube completely.

The effects of variations in treatment on the loss of CO_2 from solutions containing $NaHCO_3$ and H_2CO_3 in about the concentrations found in normal human plasma are illustrated in Table II.

9. Separation of Plasma from Centrifugated Blood.—The capillary tube *I* (Fig. 3) is connected to *J*, clamped in position as shown, and *J* and *I* are completely filled with mercury. The stopper is then removed from the centrifuge tube, and the latter is held so that the tip of *I* extends below the protecting layer of oil. With *M* at the lower level, *K* is opened and the plasma withdrawn into *J*. The pinch-cock is then placed in position on the outlet tube of *J*, *I* is removed, and *M* placed in its upper support. Samples for analysis are withdrawn as described above.

10. Preparation of Mercury and Apparatus Used for Collecting and Centrifugating Blood.—Commercial "redistilled" mercury before it was used was washed by falling from a capillary through a 1 meter column of 10 per cent nitric acid, and then through columns of distilled water. After contact with blood or serum, mercury was washed twice by shaking with distilled water, and was then passed through the nitric acid and water towers. Before use it was tested for the presence of alkali or acid by shaking a few cc. in a test-tube with water containing brom-cresol purple and phenol red. Glassware and rubber for use in contact with blood were cleaned, thoroughly rinsed with distilled water, and dried by drainage and evaporation at room temperature. When rapid drying of tonometers was necessary, they were rinsed with alcohol and ether of tested neutrality and dried in an air current.

Saturation.

We have used two processes to bring blood into equilibrium with gas mixtures of known composition. In both of them measured volumes of the gases were introduced with the blood into tonometers, which were then revolved in a water bath at 38° until equilibrium was

attained. In one process, however, the calculation of final gas tensions was based on direct gas analyses, the gas and blood being separated at the end of saturation and analyzed separately. In the other process,

TABLE II.

Loss of CO_2 from $BHCO_3$, H_2CO_3 Solutions during Standing and Centrifugating.

Change in pH was used as an index of CO_2 loss. A 0.03 M $NaHCO_3$ solution containing phenol red was saturated at 38° with air containing CO_2 at 50 mm. tension. 20 cc. portions of the solution were then transferred under oil to centrifuge tubes and treated as outlined.

No.		Initial.	Final.	Change.
		pH	*pH*	*pH*
1	An unstoppered 2 cm. (diameter) tube containing 10 cc. solution under a 1 cm. layer mineral oil was allowed to stand for 1½ hrs.	7.35	7.35	0.0
2	An unstoppered 2 cm. tube containing 10 cc. solution under a 4.5 cm. layer mineral oil was allowed to stand for 1½ hrs.	7.35	7.37	+0.02
3	An unstoppered 2.5 cm. tube containing 25 cc. solution under a 1.5 cm. layer mineral oil was centrifugated for 1 hr. and then allowed to stand for ½ hr.	7.36	7.44	+0.08
4	A stoppered 2.5 cm. tube completely filled with 25 cc. solution, under 27 cc. mineral oil, was centrifugated for 1 hr. and then allowed to stand for ½ hr.	7.36	7.52	+0.16
5	An open 2 5 cm. tube containing 25 cc. solution under a 1 cm. layer of solid paraffin was centrifugated for 1 hr. and then allowed to stand for ½ hr.	7.35	7.37	+0.02
6	A stoppered 2.5 cm. tube completely filled with 50 cc. solution and containing no oil was centrifugated for 1 hr. and then allowed to stand for ½ hr.	7.36	7.36	0.0

which was usually employed when volumes of blood exceeding 30 cc. were used, direct analyses of the gas phase were not made. The gas mixture was made up with especial accuracy, and the changes in its O_2 and CO_2 content during saturation of the blood were estimated from the amounts of these gases given off or taken up by the blood,

which was analyzed both before and after its exposure to the gas mixture. With this procedure, separation of the gas phase in condition for analysis after saturation was unnecessary.

The oxygen (commercial) used was tested for purity by absorption with pyrogallol. The CO_2 from a Kipp apparatus, was tested by absorption with KOH solution. The hydrogen, sometimes commercial, sometimes from a Kipp apparatus, was analyzed for oxygen by absorption with pyrogallol in a Haldane apparatus. Nitrogen (commercial) was analyzed for contaminating oxygen in the same manner. When air was introduced it was freed of CO_2 by passage through a tower filled with moist sodium hydroxide "shells." This treatment reduced the CO_2 content of laboratory air to 0.01 per cent or less. In detail the two procedures for saturation of the blood were carried out as follows.

First Saturation Method. Final Tensions Determined by Analysis of Gas Phase.—The tonometer used (No. 1, Fig. 3) was a modification of a form introduced by Fridericia (1920), and consisted of a relatively small vessel (5 to 30 cc.); just sufficient to hold the blood, connected by a rubber tube of 6 mm. inner diameter to a larger vessel (of about 300 cc.). A length of about 3 cm. of rubber tubing was left between the glass parts. In order to fill the tonometer with the desired gas mixture, the tonometer was connected with the gas manifold at *T* (Fig. 3), and the air was drawn out through *E*. When the saturation was to be performed at an oxygen tension lower than that of air, nitrogen or hydrogen was then twice admitted, withdrawn as completely as possible, and readmitted. It was finally again drawn out, this time only until the pressure was reduced to about half an atmosphere. From the gas burettes through *A* and *C* sufficient CO_2 and O_2 were then admitted to give the desired tensions of these gases (see Equations 3 and 5 below). Finally, nitrogen or hydrogen was admitted until atmospheric pressure was attained.

The tonometer was then rotated horizontally in the bath, the blood being so distributed between the two chambers that the ratio of blood to gas volume was about the same in both. (A rotating rack in the water bath held four tonometers at once.) The cock of the tonometer was opened near the surface of the water several times at intervals of a few minutes, until no more gas bubbles escaped. At

each opening the tonometer was held so nearly upright that all the blood drained into the lower chamber; it was redistributed before the tonometer was clamped back into place.

The time required to attain equilibrium between a gas mixture and blood is dependent on the time necessary for the tonometer contents to reach bath temperature, on the relation of gas volume to liquid surface, on the invasion coefficient of the gas (Bohr, 1905, *b*), and, also, when the gases combine with substances in the blood, on the rate of combination. Under the conditions above outlined, about 15 minutes sufficed for the production of equilibrium when only CO_2 changes were involved, while 30 to 40 minutes were required when the oxygen tension of the blood was greatly reduced by the process.

Because the Δ of Equation 3 can be estimated only approximately, the gas tensions obtained at the end of a single saturation can be only approximately predetermined. Since the exact final tensions are accurately found by gas analyses, however, approximate predetermination of them is usually all that is needed (when, for example, data are being obtained for a curve).

When, however, it was desired to bring the blood gases to an exactly predetermined final tension, exposure in the tonometer was repeated once; and, when large tension changes were involved, twice. After the first exposure the tonometer was held upright in the bath until all the blood was in the lower chamber, which was then clamped off from the upper. The gas mixture in the latter was then drawn out and replaced as described above, with a mixture of the exact tension desired.

When the final saturation was complete, the tonometer was held upright in the bath until the blood had drained as completely as possible into the lower chamber. The latter was next separated from the upper chamber by screwing two clamps upon the rubber connecting tube. One clamp was attached near each glass part, that nearest the lower chamber being screwed down first, to avoid the compression of gases over the blood that would result if the upper clamp were fastened first. The tonometer was then raised so that only the lower chamber remained in the bath, and the rubber tube was cut with scissors just below the upper clamp, leaving about 2 cm. of the tube projecting above the lower.

Into the upper, gas-containing chamber, about 50 cc. of mercury were run under pressure through the 3-way cock. The gas was thereby put under positive pressure, which facilitated drawing samples into the Haldane apparatus for analysis, and prevented the possibility of entrance of atmospheric air by leakage.

While the separated lower chamber still remained in the bath its 3-way cock was connected with a mercury leveling bulb. The clamp on the rubber outlet tube was then opened for a few seconds, while mercury was run into the chamber from below until the blood had risen into the rubber tube and displaced all the gas in the chamber. The clamp on the tube was then closed; the chamber was taken from the bath, clamped on a ring-stand, and removed for analysis of the blood.

Blood samples for analysis were drawn exactly as from the sampling tube described on page 131 (*J*, Fig. 3). In order to make the pipette tip fit tightly into the rubber outlet tube of the chamber, it was usually necessary to encircle the tip with a rubber ring cut from a section of tubing. This ring was lubricated with vaseline to facilitate fitting it into the rubber tube of the blood container.

Second Saturation Method. Final Tensions Estimated from Analyses of the Blood.—In the second method, used for larger volumes of blood than the first, a tonometer, modelled like a Barcroft gas sampling tube, with a single chamber (No. 2 of Fig. 3) of about 800 cc. capacity was employed. The volume of each tonometer, determined by weighing the water it held, was etched upon it. The gas mixture was made in the tonometer, as described in the first method, by admitting measured volumes of oxygen and CO_2, calculated by Equations 3 and 5 below. The measured volume of analyzed blood, usually about 75 cc., was drawn in through the lower cock after the significant gases had been introduced, and before the pressure was finally adjusted by admission of air, hydrogen, or nitrogen. In this case, the final total pressure at room temperature was kept 80 mm. below atmospheric by admitting the last gas through the mercury pressure regulator shown in Fig. 3. Otherwise, since the cocks were not opened during saturation, they were likely to be forced out by the pressure which developed as the gases within warmed up in the bath. The tonometers were in all cases made of Pyrex glass.

When it was necessary to know exactly the final tension at equilibrium, but not necessary that this tension be precisely at a predetermined point, one saturation was sufficient. The initial gas mixture was prepared according to Equations 3 and 5 below, to give approximately a desired final tension, and the exact final tension was calculated after analysis of the saturated blood by Equation 4.

When, however, the plan of the experiment made it desirable to fix the final tension of CO_2 or O_2, or both, at exact, predetermined points, either two or three successive saturations were performed on the same blood in different tonometers. The tension for the first saturation was calculated according to Equation 3, while for the second and third, the gases were measured into the tonometers in such proportions as to produce the exact tension desired. The total number of saturations was two, if the tension change in the blood was slight, such as the change from 45 to 40 mm. of CO_2 tension; while three equilibrations were used if the change was larger, as when blood at 40 mm. was changed to 20 or 60. Analyses of the blood were performed after each of the last two saturations. Usually both results were identical. If there was a difference, the slight effect on the final tension was calculated by Equation 4.

Transfer of Blood from Tonometer to Tonometer in Second Saturation Method.—The receiving tonometer was evacuated, and the desired amounts of CO_2 and O_2 were run in, together with enough inert gas (H_2 or N_2) to make about half an atmosphere of pressure. The two tonometers were connected below by a capillary U-tube, the delivering tonometer having been wrapped in a towel wet with water at 38° as soon as it was removed from the bath. The upper cock of the delivering tonometer was opened and the connecting tube was filled with blood by manipulation of the lower cocks. Then all but a few drops of the blood was drawn over into the receiving tonometer. Sufficient inert gas was finally admitted to raise the pressure to $B - 80$ mm.

Transfer of Blood from Tonometer to Final Container in Second Saturation Method.—One 3-way cock of the tonometer was lifted from the bath and connected with the mercury-filled receiving tube (*J*, Fig. 3), and the connections were filled with mercury. The tonometer was then inverted and placed upright in the bath, with the

mercury-filled receiving vessel below and the upper end projecting from the bath. The upper cock was opened to the air, and the blood was drawn down into the receiving tube. In some instances the exchange was quickly performed outside the bath, the tonometer having been wrapped with a towel before removal from the water.

Calculations.

The formulas used in the calculations were developed as follows:

Let p_f = final tension in mm. of mercury, of a specified gas (CO_2 or O_2) in tonometer at end of saturation.

p_i = initial tension of the specified gas in tonometer at the beginning of saturation.

Δ = increase in total (free and combined) millimolecular concentration of the specified gas in the blood caused by changing the blood from its original state to that at the end of saturation. (Δ is negative if the concentration of the gas in the blood decreases during the saturation.)

T_{tn} = absolute temperature of tonometer during saturation.

$T_{burette}$ = absolute temperature of burette from which gas is measured into tonometer.

V_{tn} = cc. total volume content of tonometer.

$V_{burette}$ = cc. volume of the specified gas measured over water in the burette, at barometric pressure and $T_{burette}$, which must be transferred to the tonometer to give therein p_i tension of the gas at T_{tn}.

V_{bl} = cc. volume of blood in tonometer during saturation.

V_{tn}-V_{bl} = cc. volume of gas space in tonometer during saturation.

B = barometric pressure in mm. of mercury.

W = vapor tension of water in mm. of mercury.

If we place the volume of CO_2 or O_2 absorbed by the blood, equal to the volume lost by the gas phase to the blood during saturation, we obtain an equation from which may be calculated the initial tension necessary to secure a given final tension or the final tension resulting from a given initial one.

The number of cc. of CO_2 or O_2, reduced to 0°, 760 mm., absorbed by the blood during saturation is equal to the product of the total cc. of blood times the volume of gas absorbed by each cc. This product is $V_{bl} \times 0.0224\Delta$. (The factor 0.0224 is the number of cc. of gas, reduced to 0°, 760 mm., contained in 1 cc. of a millimolecular solution of the gas. Consequently 0.0224Δ is the volume of gas, reduced to 0°, 760 mm., absorbed by each cc. of blood. The equations may be transformed into terms of volumes per cent of gas by substituting 0.01 for 0.0224.)

The total initial volume of the specified gas, reduced to 0°, 760 mm., in the gas space of the tonometer is $(V_{tn} - V_{bl}) \frac{p_i}{760} \frac{273}{T}$, and the final volume of the gas, at 0°, 760 mm., is $(V_{tn} - V_{bl}) \frac{p_f}{760} \frac{273}{T}$. The volume of gas, at 0°, 760 mm., lost from the gas phase to the blood during saturation is the difference between the two, or $(V_{tn} - V_{bl}) \frac{p_i - p_f}{760} \frac{273}{T}$. Placing the above two expressions equal to each other we have

$$\underbrace{0.0224\,\Delta V_{bl}}_{\text{Volume of } O_2 \text{ or } CO_2 \text{ gained by blood.}} = \underbrace{(V_{tn} - V_{bl}) \frac{p_i - p_f}{760} \frac{273}{T}}_{\text{Volume of } O_2 \text{ or } CO_2 \text{ lost by gas phase.}} \tag{1}$$

In order to find the CO_2 tension, p_i in the tonometer with which we must begin saturation to reach the desired final tension P_f we solve Equation 1 for p_i and obtain Equation 2.

$$p_i = p_f + \left(760 \times 0.0224\,\Delta \frac{T_{tn}}{273} \frac{V_{bl}}{V_{tn} - V_{bl}}\right) \tag{2}$$

When the numerical constants are combined this becomes

$$p_i = p_f + 0.0624 \frac{\Delta T_{tn}\ V_{bl}}{V_{tn} - V_{bl}}$$

$$= p_f + 19.4\Delta \frac{V_{bl}}{V_{tn} - V_{bl}} \text{ when } T_{tn} = 311° = 38°\text{C}. \tag{3}$$

Similarly if we fix the initial tension, P_i, and measure the change Δ in gas content of the blood, we can estimate the exact final tension of the specific gas at the end of equilibration as

$$p_f = p_i - 0.0624 \frac{\Delta T_{tn}\ V_{bl}}{V_{tn} - V_{bl}}$$

$$= p_i - 19.4\,\Delta \frac{V_{bl}}{V_{tn} - V_{bl}} \text{ when } T_{tn} = 311° = 38°\text{C}. \tag{4}$$

For calculation of the volume $V_{burette}$ of gas which must be measured in the burette (Fig. 3) at atmospheric pressure and transferred to the tonometer to give therein the initial tension P_i, we have used Equation 5, which is developed by placing equal to each other the expressions indicating the volume of gas measured in the burette and in the tonometer, respectively, both being reduced to 0°, 760 mm. We then have

$$V_{burette} \times \frac{\text{B-W}}{760} \times \frac{273}{T_{burette}} = (V_{tn} - V_{bl}) \frac{p_i}{760} \frac{273}{T_{tn}}, \text{ whence}$$

$$V_{burette} = (V_{tn} - V_{bl}) \frac{p_i}{B\text{-}W_{burette}} \frac{T_{burette}}{T_{tn}}, \text{ or}$$

$$= \frac{p_i (V_{tn} - V_{bl})}{T_{tn}} \frac{T_{burette}}{B\text{-}W_{burette}} \tag{5}$$

Solving Equation 5 for P_i we obtain in Equation 6 the tension at bath temperature given by the gas volume, $V_{burette}$, measured into the tonometer.

$$p_i = V_{burette} \frac{T_{tn}}{V_{tn} - V_{bl}} \frac{B\text{-}W_{burette}}{T_{burette}} \tag{6}$$

We have found it convenient to use Equation 5 in the second of the two forms given above, for the reason that it places in one group the factors p_i, T_{tn}, and $(V_{tn} - V_{bl})$ which are independent of temporary room conditions and can therefore be calculated in advance, while the factors $T_{burette}$ and $(B - W_{burette})$, which must be determined at the moment of measurement, are placed together in a second group.

Approximate Estimation of Δ for Calculation of Initial Tensions of First Saturation.—This estimate was made by means of the average absorption curve of the kind of blood used. The manner in which the estimate was made is shown by an example. In the venous blood drawn from the horse used in most of our experiments the CO_2 tension was constantly in the neighborhood of 45 mm. The average absorption curve of the animal's blood showed that changing the CO_2 tension produced approximately the following changes in CO_2 content:

p_f	CO_2 content.	Δ
mm.	mM.	mM.
20	15.0	−6.6
40	20.5	−1.1
45	21.6	0.0
60	23.5	+1.9
80	25.2	+3.6

While the CO_2 content of the blood as drawn varied at times considerably from 21.6 mM., the values of Δ caused by given changes in tension remained fairly constant.

Exact Determination of Δ for Calculation of Final Gas Tensions.—This determination was made by comparison of the analyses of the blood after each of the last two saturations.

Example:

Equilibration No.	CO_2 content of blood.	Δ
	mM.	mM.
2	21.6	−0.4
3	21.2	

Limits of Error in Determination of Final CO_2 and O_2 Tensions.

1. By Analysis of Gas Phase at End of Saturation (First Method).—The analytical error of CO_2 and O_2 determinations by the Haldane apparatus is ordinarily about ± 0.02 volumes per cent. The tension corresponding to this at 760 mm. and 38° is $\frac{0.02}{100}(760 - 49) = 0.14$ mm. of mercury. It appears probable that the error of estimating gas tensions produced by the "First saturation method" may be kept below 0.2 mm., since errors are practically excluded in separating the gas phase for analysis. As a matter of fact, results shortly to be published indicate that this degree of accuracy can be obtained quite consistently.

2. By Analysis of Blood Before and After Saturation with Known Gas Mixtures (Second Method).—The final tension is determined by Equation 4, which for 38° is

$$p_f = p_i + 19.4\,\Delta\,\frac{V_{bl}}{V_{tn} - V_{bl}}$$

In determining the possible error in calculating P_f, we estimate and add the maximum errors in the experimental determinations of P_i and of the value $19.4\ \Delta\ \frac{V_{bl}}{V_{tn} - V_{bl}}$, respectively, the latter value representing the correction which must be applied to P_i as the result of the loss or gain of significant gas by the blood during saturation.

p_i is determined by the amount of gas measured into the tonometer. From Equation 5 we have $p_i = \frac{V_{burette}}{V_{tn} - V_{bl}} \times (B - W_{burette}) \times \frac{T_{burette}}{T_{tn}}$.

At the usual conditions, *viz.* $T_{burette} = 20° + 273$, $T_{tn} = 38° + 273$, $B = 760$, $B - W = 743$, $V_{tn} = 800$, $V_{bl} = 75$, we have $p_i = V_{burette} \times 1.17$. 1 cc. of gas, measured in the burette (Fig. 3), therefore corresponds to a tension of about 1.2 mm. of mercury in the tonometer. The gas can be measured with an error not exceeding 0.10 cc., so that p_i can be fixed within about $0.10 \times 1.2 = 0.12$ mm. by measurement. The errors in the other factors, *viz.* $(V_{tn} - V_{bl})$, $(B - W)$, and $\frac{T_{burette}}{T_{tn}}$ are relatively so small as to be negligible.

The error in determining the value of the term $19.4\ \Delta\ \frac{V_{bl}}{V_{tn} - V_{bl}}$ may be estimated as follows: In our experiments the blood occupied about one-tenth as much space as the gases in the tonometer, $\frac{V_{bl}}{V_{tn} - V_{bl}} = 0.1$. Substituting 0.1 for the factor $\frac{V_{bl}}{V_{tn} - V_{bl}}$ in Equation 4, for 38°, we have $p_f = p_i - 1.94\ \Delta$. That is, a change of 1 mM in the value of Δ for CO_2 or O_2 causes a change of about 1.9 mm. in the tension of the gas at 38°. The analytical error of our blood gas determinations is about $\pm$ 0.05 mM.; and Δ represents the difference between two determinations, so that its possible error would be twice as great, or $\pm$ 0.1 mM. The latter would cause an error of 1.9×0.1, or about 0.19 mm. in the calculation of the value, $1.94\ \Delta\ \frac{V_{bl}}{V_{tn} - V_{bl}}$.

The total error in the determination of the final tension in the manner indicated by the equation $p_f = p_i + 19.4\ \Delta\ \frac{V_{bl}}{V_{tn} - V_{bl}}$ we

therefore estimate as approximately 0.12 + 0.19 = 0.3 mm. of O_2 or CO_2 tension. This is of the same order of magnitude as the error involved in estimating the final tension by analysis of the tonometer gas, discussed above.

The error in the calculation from Δ may be reduced in either of two ways. (1) Since the error is chiefly due to the factors involved in the term $\Delta \frac{V_{bl}}{V_{t_n} - V_{bl}}$, it may be reduced by reducing V_{bl}, the volume of blood used, and therefore the factor $\frac{V_{bl}}{V_{t_n} - V_{bl}}$.

TABLE III.

Sample.	CO_2 tension calculated from volume of CO_2 measured into tonometer.	CO_2 tension calculated from analysis of tonometer gas.
	mm. Hg.	*mm. Hg.*
1	24.42	24.35
2	25.23	25.01

(2) When the final tension has been estimated by Equation 4, the blood may be saturated once more, at exactly this tension. The error is thereby practically reduced to that involved in fixing p_i, which is only 0.1 mm. of tension. This procedure has been followed when maximum accuracy has been required in the saturation of large volumes (50 – 100 cc.) of blood.

The following experiment indicates that with the apparatus used the two methods for fixing initial tensions agree within their limits of error:

Into two tonometers, in which the pressure had been reduced to about 60 mm., measured amounts of CO_2 were introduced from burette *A* (Fig. 3). Air was admitted until the pressure in the tonometers was brought to atmospheric. Samples of the gas mixture were then displaced from the tonometers into a Haldane air analysis apparatus and analyzed for CO_2. CO_2 tensions calculated from the volume of CO_2 introduced (Equation 5) and the tensions calculated from the results of the gas analyses, respectively, are shown in Table III.

That the final tension can be set with a similar degree of accuracy is indicated by the constancy of the final CO_2 contents obtained

TABLE IV.

Formula No.	Use of formula.	Formula in terms of $[H^+]$ and K'.	Formula in terms of pH and pK'.
1	Calculation of $[H_2CO_3]$ from p.	mM. $[H_2CO_3] = \frac{\alpha p}{760 \times 0.0224}$ $= 0.0587\,\alpha\,p$	
2	Calculation of $[BHCO_3]$ from $[CO_2]$ and p.	mM. $[BHCO_3] = [CO_2] - 0.0587\,\alpha\,p$	
3	Calculation of $[H^+]$ or pH from $[CO_2]$ and p.	$[H^+] = K' \frac{0.0587\,\alpha\,p}{[CO_2] - 0.0587\,\alpha\,p}$	$pH = pK' + \log \frac{[CO_2] - 0.0587\,\alpha\,p}{0.0587\,\alpha\,p}$
4	Calculation of K' or pK' from $[H^+]$ or pH and $[CO_2]$.	$K' = [H^+] \frac{[CO_2] - 0.0587\,\alpha\,p}{0.0587\,\alpha\,p}$	$pK' = pH - \log \frac{[CO_2] - 0.0587\,\alpha\,p}{0.0587\,\alpha\,p}$
5	Calculation of CO_2 tension from pH and $[CO_2]$.	$p = \frac{[CO_2]}{0.0587\,\alpha\left(\frac{K'}{[H^+]} + 1\right)}$	$p = \frac{[CO_2]}{0.0587\,\alpha\,(10^{pH - pK'} + 1)}$
6	Calculation of $[BHCO_3]$ from pH and $[CO_2]$.	$[BHCO_3] = [CO_2] \frac{1}{1 + \frac{[H^+]}{K'}}$	$[BHCO_3] = [CO_2] \frac{1}{1 + 10^{pK' - pH}}$

* In order to use formulas with $[CO_2]$, $[BHCO_3]$, and $[H_2CO_3]$ expressed in terms of volumes per cent of CO_2 instead of mM. concentration, replace the factor 0.0587 by the factor $\frac{100}{760}$, or 0.1316.

in the accompanying second paper of the series. In the horse blood used a deviation of 0.7 volume per cent or 0.3 mM. in CO_2 content corresponds to a deviation of 1 mm. in CO_2 tension.

Formulas for Calculating pH, $[H^+]$, p_{CO_2}, $[BHCO_3]$, and $[H_2CO_3]$ from Data Usually Obtained by Direct Determination.

We have used certain rearrangements of Henderson's and Hasselbalch's equations so frequently that it appears desirable, in order

TABLE V.

Values of Constants for Formulas of Table IV.

	α	0.0587α	0.1316α	K′	pK′
Water	0.555*	0.0326	0.0730	†	†
Serum or plasma	0.541*	0.0318	0.0712	7.2×10^{-8}	6.14‡
Blood	0.511*	0.0300	0.0672	6.5×10^{-8}	6.18‡
12 per cent § hemoglobin + 30 mM. $NaHCO_3$	0.531‡	0.0312	0.0699	6.5×10^{-8}	6.18‡

* Bohr, 1905.

† Variable with $NaHCO_3$ concentration. See Hasselbalch, 1917.

‡ Data to be published. (K′ and pK′ determined on horse blood.)

§ Containing hemoglobin sufficient to bind 16 volumes per cent of oxygen. Millimolar hemoglobin concentration $= \frac{16}{2.24} = 7.15$ mM., assuming that 1 molecule of oxygen combines with 1 molecule of hemoglobin.

to avoid subsequent repetition, to record them here in connection with the description of technique.

The constants are given in terms of millimolecular (mM.) concentration, rather than in volumes per cent of gas, for the reason that comparison of concentration changes not only in oxygen and carbon dioxide, but also in electrolytes, such as chlorides and alkali protein compounds, has been necessary in the studies outlined, and a single unit of concentration that can be used throughout is desirable.

The following symbols are used:

$[CO_2]$, $[BHCO_3]$, $[H_2CO_3]$ = millimolecular concentration of total CO_2, $BHCO_3$, and H_2CO_3, respectively.

α = solubility coefficient of CO_2 at 38°.

p = tension of CO_2 in millimeters of mercury.

K′ = constant by which the ratio $\frac{[H_2CO_3]}{[BHCO_3]}$ must be multiplied to give $[H^+]$. Theoretically $K' = \frac{K}{\gamma}$ where K is the dissociation constant of H_2CO_3, γ the fraction of $BHCO_3$ dissociated into B^+ and HCO'_3 (Hasselbalch, 1917).

$pK' = -\log K'$ (Hasselbalch, 1917).

For the constants α, K′, and pK′, the values in Table V have been used.

In Formula 2 the $[BHCO_3]$ is calculated by subtracting from the total $[CO_2]$ the $[H_2CO_3]$, which is calculated from the CO_2 tension according to Formula 1.

Formula 3 is Henderson's familiar $[H^+] = K'\frac{[H_2CO_3]}{[BHCO_3]}$ with $[H_2CO_3]$ and $[BHCO_3]$ calculated according to Formulas 1 and 2.

Formula 4 is obtained by obvious rearrangement of Formula 3.

Formula 5 is obtained by solving Formula 3 or 4 for p.

Formula 6 is obtained by substituting the value of p from Formula 5 for p in Formula 2.

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STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

II. THE REVERSIBILITY OF THE EFFECTS OF CHANGES IN CO_2 AND O_2 TENSIONS ON THE CO_2 CONTENT OF DEFIBRINATED HORSE BLOOD.

BY JOHN P. PETERS, GLENN E. CULLEN, AND J. HAROLD AUSTIN.

(From the Hospital of The Rockefeller Institute for Medical Research.)

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Haggard and Henderson[1] have stated that when the CO_2 tension of dog blood, either defibrinated or oxalated, has been reduced to less than about 20 mm. the blood undergoes an irreversible, or at least not readily reversible, change characterized by a diminution of its CO_2 capacity. When this blood was again brought into equilibrium with a gas mixture with a higher CO_2 tension it took up less CO_2 than before its exposure to the low CO_2 tension. They found this irreversible change more consistently in defibrinated than in oxalated blood.

Reversibility of the Effect of Low CO_2 Tension.—In order to determine whether such an irreversible change occurred in defibrinated horse blood the following experiments were carried out. Fresh horse blood was defibrinated by stirring under oil and was then strained through gauze under oil. The blood was divided into two portions. One of these was first exposed to a low tension of CO_2 and then saturated at 60 mm. CO_2 tension. The other was at once saturated at 60 mm. CO_2 tension. The CO_2 contents of the two were then compared. The "second saturation method" described in the first paper of these studies was used.[2] The blood gases were determined by the blood gas apparatus described by Van Slyke and Stadie,[3] as the constant volume apparatus was not yet in use.

[1] Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1920–21, xlv, 209.

[2] Austin, J. H., Cullen, G. E., Hastings, A. B., McLean, F. C., Peters, J. P., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liv, 121.

[3] Van Slyke, D. D., and Stadie, W. C., *J. Biol. Chem.*, 1921, xlix, 1.

The first portion was exposed at 38° in a tonometer which had been filled with air free from CO_2. The final CO_2 tension in this tonometer, due to CO_2 evolved from the blood, was calculated to be 15 ± 2 mm. This tension was further confirmed by the location of the CO_2 content of the blood from this tonometer on the CO_2 absorption curve of the blood. The blood was then passed through a series of three tonometers at 38°. Into the first was introduced enough CO_2 to produce a CO_2 tension of 60 mm. at 38° after allowance had been made for the CO_2 which would be taken up by the blood. Into the two succeeding tonometers was introduced the exact amount of CO_2 required to give a tension of 60 mm., assuming no further removal of CO_2 by the blood. The blood was analyzed for CO_2 after exposure in each of the last two tonometers.

The second portion of blood was also passed through a series of four tonometers at 38° for the same periods of time. Into each of these tonometers was introduced the amount of CO_2 required to give a tension of 60 mm. at 38°. (The CO_2 placed in the first tonometer was corrected for the CO_2 which it was estimated the blood would absorb.) The blood from the last two tonometers was analyzed for CO_2.

The above experiment was duplicated on a different sample of blood from the same horse. The results of the two experiments are given in Tables I and II.

Reversibility of the Effect of Low Oxygen Tension.—Blood was withdrawn under albolene from the jugular vein of a horse, defibrinated by gentle stirring with a glass rod, and filtered through gauze, under a layer of albolene. It was divided into two portions (Nos. 1 and 2) of 50 cc. each. One of these (No. 2) was exposed in a tonometer at 38°C. to 40 mm. of CO_2 in commercial nitrogen (No. 2 a), and then transferred to a second tonometer and exposed to a mixture of 40 mm. of CO_2 and 13 mm. of oxygen in commercial nitrogen. A sample was then removed and analyzed for CO_2 and oxygen (No. 2 b). The remainder was transferred successively to three more tonometers (Nos. 2 c, 2d, and 2 e) in each of which it was exposed to 40 mm. of CO_2 in air. (In making up the gas mixtures in tonometers 2 a and 2 c an allowance was made for the change of CO_2 and oxygen content in the blood.) Samples were removed from Nos. 2 d and 2 e and

analyzed for CO_2. The amount of CO_2 in both was identical, demonstrating that complete gaseous equilibrium had been established.

TABLE I.

Saturation.	Exposed to low CO_2 tension.		Not exposed to low CO_2 tension.	
	CO_2 tension.	CO_2 content.	CO_2 tension.	CO_2 content.
	mm.	*vol. per cent*	*mm.*	*vol. per cent*
First	15*	33.0	60*	
Second	60*		60	
Third	60	53.7	60	54.4
Fourth	60	53.7	60	54.45

* Approximate.

TABLE II.

Saturation.	Exposed to low CO_2 tension.		Not exposed to low CO_2 tension.	
	CO_2 tension.	CO_2 content.	CO_2 tension.	CO_2 content.
	mm.	*vol. per cent*	*mm.*	*vol. per cent*
First	15*		60*	
Second	60*		60	
Third	60	53.1	60	53.8
Fourth	60	53.8	60	53.5

* Approximate.

TABLE III.

No.	Treatment of blood.	CO_2 content.	O_2 content.
		vol. per cent	*vol. per cent*
1 e	Exposed in four successive tonometers to 40 mm. of CO_2 in air.	48.3	14.8
2 b	Exposed in two successive tonometers to 40 mm. of CO_2 and 13 mm. of O_2.	51.7	5.1
2 e	Same blood exposed in two more successive tonometers to 40 mm. of CO_2 in air.	48.1	14.8

Meanwhile the other specimen (No. 1) had been passed through the same number of tonometers, in each of which it had been exposed to 40 mm. of CO_2 in air. Analysis of the last specimen (No. 1 e)

for CO_2 and O_2 gave results identical with those obtained from specimen No. 2 e, which had first been exposed to CO_2 in nitrogen. Similar results have been obtained in other experiments.

It is evident from these experiments that the reduction of the CO_2 tension of defibrinated horse blood to as little as 15 mm. produces no irreversible change in the carbon dioxide capacity of the blood. There appears to be no doubt of the reversibility of the reactions between 15 and 60 mm. of CO_2 tension. It seems likely, as Evans[4] suggests, that the irreversible fall in CO_2 capacity, observed by Haggard and Henderson[1] was due to the formation of acid in the blood. This acid formation is very rapid in dog blood[2] and is accelerated, according to Evans, by reduction of CO_2 tension (increase of pH).

Our results further indicate that the effect of reduced oxygen tension on the CO_2 capacity of blood is entirely reversible.

[4] Evans, C. L., *J. Physiol.*, 1922, lvi, 146.

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THE SIGNIFICANCE OF COLOSTRUM TO THE NEW-BORN CALF.

BY THEOBALD SMITH, M.D., AND RALPH B. LITTLE, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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The high mortality of calves during the 1st week of life has been the subject of researches for a number of decades. The chief symptoms preceding death are diarrhea, whence the name scours, inflammation of the umbilical stump (omphalitis), and multiple exudative arthritis. The concensus of opinion today is that there is a more or less close relation among these clinical manifestations and that, as pointed out by C. O. Jensen,[1] the chief infecting agents are races of *Bacillus coli*. In the latest summaries of handbooks covering this subject the emphasis is placed upon the infectious agents and all the measures that are recommended involve isolation, cleanliness, disinfection, and specific serum treatment.

Running parallel with these investigations but hardly influenced by them are studies on the transmission of immune bodies from mother to fetus and nursling. The fundamental studies of Ehrlich on ricin and abrin immunity transmitted to mice in the milk of immune mothers were followed by a series of investigations involving both normal and artificially induced antibodies in the mother's milk. These showed that not all species act alike, that some may transmit antibodies to the fetus *in utero*, others only in the milk.

The difficulty experienced in keeping calves alive which had not received colostrum from the mother led to a more thorough study of the effect of withholding this first milk. The significance of colostrum to the new-born calf is not set forth in medical or veterinary

[1] Jensen, C. O., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, vi, 121.

literature with any definiteness. This fluid is generally assumed by some as necessary to cause evacuation of the fetal fluids and solids from stomachs and intestines. Others think that a cathartic like castor oil may replace it. In formulating measures for preventing calf scours Hutyra and Marek,[2] probably basing themselves on the work of C. O. Jensen[3] who stated in 1905 that calves die of enteritis if fed cooked or pasteurized milk during the first 24 hours of life, advise feeding the dam's colostrum for a day before heated milk is fed. They suggest that the fore milk be rejected since it might be infected. In the carrying out of the Bang system for the control of bovine tuberculosis, some have withheld colostrum, others have allowed the calf to suckle the dam immediately after birth, although the original method permits the calf to remain with the dam until it has taken the first milk.

Considerable work on the problem of feeding new-born calves has been done by W. L. Williams and associates.[4] Although the factor of colostrum entered, it was complicated with the use of scours sera and the boiling or pasteurizing of milk. No definite experiments on the simple exclusion of colostrum from the diet and the association with these of adequate controls by which the unknown, uncontrolled factors might be balanced are reported.

J. Traum[5] takes up the question of raising calves from a tuberculous herd by excluding the colostrum and milk of the reacting dam. Traum does not, however, state how many calves did not receive colostrum or milk immediately following the colostric period, for he writes that in the herd in which the experiment was going on "there were usually about 25 fresh cows yielding sufficient colostrum to frequently supply the calves born in reacting herd." Without doubt Traum did raise calves without colostrum, but he does not state the number as compared with those that did receive it nor give any subsequent history of the calves, nor does he report any losses by the way.

So far as it has been possible to examine the literature, the statement is warranted that no experiments dealing exclusively with the

[2] Hutyra, F., and Marek, J., Spezielle Pathologie und Therapie der Haustiere, Jena, 4th edition, 1920, i, 163.

[3] Jensen, C. O., *Z. Thiermed.*, 1905, ix, 321.

[4] Williams, W. L., et al., *Rep. New York State Vet. College*, 1914–20.

[5] Traum, J., *J. Am. Vet. Med. Assn.*, 1921, lix, 755.

withholding of colostrum have been made which at the same time include a number of controls sufficient to eliminate highly virulent epizootics of scours. For this reason it has seemed justified to record the following experiments with a certain number of details concerning the individual calves gathered together in an appendix.

EXPERIMENTAL WORK.

In order to balance as far as possible the many unknown factors entering into the undertaking, two experiments were carried on simultaneously. One group of calves was to have colostrum, the other not. The calves were obtained from the same large herd. To make sure that none that had suckled the mother soon after birth should be included in the group not having taken colostrum, only those born during the day and definitely known to the attendant to be without food were chosen. These were removed with as little exposure as possible to the Department units, a distance of 1½ miles. The calf was covered with a blanket, placed in a crate, and transported in a protected autotruck. The units were artificially heated. In the early experiments the calves were gently rubbed with cloths until nearly dry. Later they were washed with hot water containing some soda to soften the mucus adhering to the hair and thoroughly rubbed until dry. The umbilicus in later cases was snipped off if longer than 1 inch, gently manipulated to remove fluids, dipped in 0.1 per cent bichloride of mercury, and dusted with boric acid powder.[6] In some the bichloride was omitted. The milk was fed soon after milking and was raw in all cases.

The group which took colostrum comprised ten calves. All of these survived the danger period and were kept various periods of time thereafter. Three died unexpectedly after 25, 38, and 45 days respectively (Nos. 757, 694, and 715). The protocols indicate that death was most probably due to some kind of poison and experiments are under way to clear up if possible the cause of death. Whatever this cause may have been, the evidence is quite conclusive that there was no ordinary infection involved, since in the two from whose organs cultures were made bacteria could not be demonstrated even though in one case (No. 715), death having occurred early Sunday

[6] Williams, W. L., *Rep. New York State Vet. College*, 1913–14, 163.

morning, the autopsy was not made until after 28 hours refrigeration. A condition common to the three animals was the presence of punctiform hemorrhages throughout the intrathoracic portion of the thymus.

Of the second group of 12 calves which received no colostrum, nine died and three survived. These may be grouped according to the length of life. Seven of the calves died within 6 days. Thus four (Nos. 663, 685, 699, and 566) died within 3 days, one (No. 895) in 3½ days, one (No. 894) in 4½ days, and one (No. 665) in 5½ days. One (No. 682) not included in the figures was too weak to take food and died within 2 days.

Before considering the gross appearances presented at autopsy, it should be stated that in new-born calves minute hemorrhages are almost regularly encountered in certain organs. They are rarely absent from the auriculoventricular valves of the heart. These have been made the subject of special study by Laura Florence.[7] The mucosa of the leafy or expanded portion of the fourth stomach is regularly sprinkled over with petechiæ. The area involved varies from a few centimeters to the entire stomach. In some cases the hemorrhages are deep, in others superficial. Each spot is then capped with a bit of digested blood. Notably the pyloric valve is the seat of minute hemorrhages and in calves several months old shallow ulcers are occasionally still present on this valve. Less frequently the lowest 3 to 4 inches of the rectal mucosa is deeply congested and blood is now and then seen on the discharges. The ileocecal valve in rare instances is permeated with hemorrhages. In a few cases the mucosa of the rumen is involved in hemorrhages. If we except the heart valves, the hemorrhages, so far as studied, may be tentatively considered as mechanical in origin and due to conditions arising during parturition. Wherever in the present paper these hemorrhages are mentioned, the above facts should be borne in mind.

The appearances at autopsy differed somewhat from animal to animal and were due to differences in the blood content of the organs. The mucosa of the small intestine was in some instances pale, in others deeply congested in parts or throughout. The large intestine also varied in this respect. In all cases the small intestine was more deeply congested than the large. The other organs most involved were the kidneys. These varied from a nearly normal coloration and consistency to an intense congestion of the entire organ and a much firmer consistency. The urine taken from the bladder was free from blood or hemoglobin and contained only a trace of albumin

[7] Florence, Laura, *Am. J. Dis. Child.*, 1922, xxiii, 132.

with one or two exceptions. The liver cells usually contained more or less visible fat. The spleen was normal in size and flabby. The thoracic organs presented nothing characteristic.

Coming to the bacteriology of these cases we meet a definite condition in all animals. The spleen, liver, and kidneys contained large numbers of *Bacillus coli*. Each loopful of tissue fluid contained hundreds of bacteria. There was undoubtedly to a certain extent

TABLE I.

The Results of Feeding and Withholding Colostrum.

No. of calf.	Colostrum fed +; not fed −.	Source of dam	No. of pregnancy.	Source of milk fed.	Date of birth.	Died, killed, or sold.	Remarks.
663	−	Eastern.	1	601	Oct. 4	Died, Oct. 6.	*B. coli* septicemia.
664	+	Native.	1	600, 601	" 10	Sold, Nov. 2.	
665	−	Western.	1	601	" 11	Died, Oct. 16.	*B. coli* septicemia.
669	+	Native.	1	601	" 18	Killed, Feb. 13.	Normal.
680	+	"	1	678	Nov. 1	Sold, Dec. 10.	
681	−	Eastern.	3 (?)	?	" 2	Killed, Nov. 5.	*B. coli* septicemia.
685	−	Native.	3	?	" 9	Died, " 11.	" " "
687	+	Western.	1	679	" 9	Sold, Dec. 10.	
694	+	Eastern.	1	679	" 25	Died, Jan. 2.	No cultures. Poison (?).
697	−	"	3	679	" 29	Sold, Feb. 2.	
698	+	Native.	4	678	Dec. 6	" " 2.	
699	−	"	3	678	" 6	Died, Dec. 8.	*B. coli* septicemia.
715	+	"	1	712	Jan. 5	" Feb. 19.	Cultures sterile. Poison (?).
717	−	Western.	1	678	" 11	" Jan. 22.	*B. coli* septicemia.
716	−	Native.	3	712	" 11	Killed, Mar. 16.	Normal. Cultures sterile.
718	+	Western.	1	678	" 12	Sold, Feb. 2.	
757	+	Native.	3	678	" 23	Died, " 17.	Cultures sterile. Poison (?).
759	−	"	2	678	" 25	Killed, " 21.	Miscellaneous bacteriemia.
893	+	"	1	712	Feb. 9	" Mar. 28.	Normal.
894	−	"	1	712	" 9	Died, " 13.	*B. coli* septicemia.
895	−	Eastern.	4	678	" 10	" " 13.	" " "
566	−	Native.	1	678	" 10	" " 12.	" " "

multiplication after death but, as will be seen farther on, these bacilli are present before natural death in abundance. Other organs were not cultured except in several cases certain joints which also yielded *Bacillus coli.*

The calf (No. 682) which died within 2 days and whose stomachs contained no food presented the same *Bacillus coli* septicemia.

No. 681 is of interest. On the 4th day of life it was too weak to get up and take its food. It would have presumably died during the night and it was therefore killed. The kidneys were firmer than normal and deeply congested. Cultures showed the presence of large numbers of *Bacillus coli* in the spleen, liver, and kidneys.

One calf (No. 717) died when 11 days old of a *Bacillus coli* septicemia. Cultures from one tarsal joint contained only *Bacillus coli.*

One calf (No. 759) which survived became lame. Several joints were involved. It was killed when 27 days old. There was a purulent fluid and a fibrin mold in one joint. The kidneys presented numerous whitish sclerotic foci. Cultures from the spleen remained sterile. Those from the kidneys and liver represented mixed infections, those from the joint exudate pure *Bacillus coli.* Of the two remaining calves, one was sold to the butcher in fine condition when 2 months and 3 days old. The other was killed when 2 months and 5 days old. The organs presented no abnormalities.

In Table I the cases are arranged according to the dates of birth. It shows that the calves deprived of colostrum and the controls were well balanced as to season.

DISCUSSION.

The data presented in the foregoing cases lead to certain definite inferences. The calf deprived of colostrum lacks something which permits intestinal bacteria to invade the body and multiply in the various organs. The rapidity and duration of this multiplication determine the fate of the calf. In most cases a rapidly fatal septicemia is the result. When the resistance is greater life may be prolonged or the animal survive indefinitely. The surviving animal may completely subdue the invading bacteria and develop into a normal calf or else localizations arise which involve, in the cases

here presented, the joints or the kidneys or both organs as in No. 759. The joint lesions are due to a fibrinous and purulent exudation. The bacteria present belong to *Bacillus coli*. The end-result of the localization in the kidneys is a fibrosis limited to the cortex with localized destruction of the secreting tissue. This occurs in foci of varying extent. The largest seen were about 1 cm. in diameter. This pathological condition, which was observed in 1917 associated with pneumonia due to *Bacillus actinoides*,[8] may now be considered as partly cleared up and it remains to study the early stages leading to fibrosis. The precise etiological factor may be *Bacillus coli* or some organism associated with it.

The bacteria isolated from the various cases and which were present in very large numbers in those that died and in fairly large numbers in those that were killed and cultured within 2 hours belong to that variety of *Bacillus coli* which is non-motile, indole-producing, and which fails to act on saccharose. This particular type of *Bacillus coli* has been under observation since 1917 and has been frequently isolated from the small intestines of calves dying in the first few days of life.

The experiments described are in a sense fundamental and many subsidiary questions are left unanswered. One of these is the effect of the ordinary milk of the different cows fed, whether protective and if so to what degree. In the table, the cow's milk fed is indicated by a number and it will be seen by consulting this that the source of the milk is not a determining factor in the experiment. Another question to be answered is whether it might not be possible by using extreme and unremitting care to raise most calves deprived of colostrum and how many of these would develop scours, joint lesions, omphalitis, or pneumonia in due time.

In general it may be concluded that the function of the colostrum is essentially protective against miscellaneous bacteria which are harmless later on when the protective functions of the calf have begun to operate and accumulate energy. There appears to be no function inherent in colostrum which controls development or growth or which is essential to the starting of the mechanism of digestion,

[8] Smith, T., *J. Exp. Med.*, 1921, xxxiii, 441.

since calves not having had colostrum appear to do as well as the others when the infection has been overcome.

The results obtained clear up much of the mystery surrounding the mortality and morbidity of very young calves. They explain why disease may appear when there is no evidence of introduction from without, and why the causes of disease of young calves up to 2 months old have been referred to the first days of life and to prenatal infection, especially where infectious abortion is prevalent. They moreover point out why so many different kinds of bacteria have been regarded as responsible for calf scours. Obviously the most invasive of the flora of any herd will dominate the bacteriology and different organisms will predominate in different territories unless the interchange of animals is very active, in which case the same flora will probably be found in many herds. The results also explain mixed infections, since the portal of entry from the intestinal tract is open.

These results furthermore show the inadequacy of all attempts at prevention based on keeping the invading bacteria away. These live in the normal cow, possibly some in the udder itself, and must be considered ubiquitous. They also demonstrate the futility of administering specific serum unless the dominating pathogenic organisms of the herd are known and used in its preparation.

It is not claimed as a result of these experiments that all colostrum-fed calves will resist the various forms of disease described, for we know that they prevail in spite of normal feeding at birth. There may be degrees of virulence in the intestinal, respiratory, and udder flora of cows against which normal feeding is quantitatively inadequate. It so happened that in the experiments reported no highly virulent types were present and therefore the dividing line between the calves protected by colostrum and those not so protected was unusually well defined.

CONCLUSIONS.

All of ten calves which were permitted to take colostrum after birth survived. Eight out of twelve calves which did not get colostrum died and one was killed moribund. One calf, killed on the 27th day, harbored miscellaneous bacteria in its organs. The kidneys

were sclerotic and one joint diseased. Of the remaining two calves, one had transitory joint troubles, the other rhinitis. One was sold and the other killed when 2 months old. In the latter the organs were normal and sterile.

Notes on the Individual Calves.

Calf 566.—Male, born Feb. 10, 1922, at 2.30 p.m., of a native cow in her second pregnancy. Calf removed from the dam immediately after birth and taken to the Department buildings at 4 p.m. There it was dried off by gentle friction with cloths. The umbilical cord was treated with bichloride of mercury and powdered boric acid. Fed 1½ lbs. milk (of Cow 678) from a bottle in evening.

Feb. 11. Calf fed three times today, drinking in all 4½ lbs. of milk. Temperature 39.0–39.5°C.

Feb. 12. Temperature 39.2°C. early. Calf drank first meal. Died about 11 a.m. Refrigerated at once until Feb. 13. The autopsy showed little of significance except the following.

The fourth stomach contained a milk curd the size of a small apple, some smaller clots, and milky fluid. Punctiform hemorrhages in leafy portion. Rectum deeply congested. Liver cells contain much fat. Extensive subendocardial extravasations of blood. Urine taken from bladder contains a trace of albumin. Both metatarsal joints contain some light yellowish fluid and soft mucoid flakes consisting largely of polynuclear leucocytes.

Cultures from spleen, liver, and kidneys are overgrown with a heavy glistening layer which in subsequent dilution cultures proves to be the saccharose-non-fermenting *B. coli*. One of several tubes inoculated with the exudate from the tarsal joint contains numerous colonies of the same organism.

Calf 663.—Born Oct. 4, 1921, at 11.50 a.m., of an eastern cow. First calf since introduction into herd. Calf not allowed to suckle the dam, and removed to Department unit at 1.35 p.m. Fed 2½ lbs. of milk from Cow 601 at 5.30 p.m. Abundant discharge of meconium during the night. Fed next morning. The discharges became softer and were yellowish in color.

Oct. 6. The calf was weak, unable to get up, and still scouring. Respirations shallow, about 60 per minute. Opaque, whitish, mucoid discharge from left nostril. Calf died at 11 a.m.

Autopsy.—Weight 67 lbs. Umbilical cord shrunken to a dried black tape-like body. Hemorrhagic discoloration of sheath and suspensory ligaments of umbilical artery stumps. Umbilical vein patent within abdomen to liver. Contents a normal blood clot. Beneath epicardium around base of heart, some hemorrhages. Hemorrhages within heart valves (auriculoventricular).

Digestive tract: Rumen normal. Fourth stomach contains white curds and some opalescent fluid. Leafy portion sprinkled with faded petechiæ. Pyloric portion and valve deeply congested. Uniform deep congestion of entire length

of small intestine. Bits of mucosa examined microscopically show complete injection of the network of capillaries in the villi. Large intestine congested but less so than the small. Liver shows considerable intracellular fat. The spleen is flabby, somewhat congested. Kidneys deeply congested. Urine taken from bladder is faintly clouded, yellow, slightly acid. Contains a trace of albumin and no sugar. Specific gravity 1.026.

Cultures from spleen and liver indicate large numbers of *B. coli* evidently pure. From the contents of a loop of the small intestine dilutions spread on agar show the same type of colonies. Those from a liver culture replated found to be colon bacilli non-motile, not fermenting saccharose.

Calf 664.—Born Oct. 10, 1921, at 5.20 p.m., first calf of a native cow, A 945. The dam had been vaccinated with a living culture of *B. abortus* before being bred. The parturition was normal and a guinea pig inoculated with material from a uterine swab was negative for *B. abortus*.

The calf remained with dam until 8.20 a.m. next day when it was removed to an isolation unit at the Department and fed milk from Cows 601 and 600. The temperature taken twice daily fluctuated between 38° and 39°C. The calf remained well and was sold Nov. 2.

Calf 665.—Born Oct. 11, 1921, at 2.50 p.m., of a midwestern cow introduced into the herd Sept. 27. Calf removed immediately after birth to a Department unit at 3 p.m. and fed milk from Cow 601.

Oct. 12. Calf drank 5 lbs. of milk in morning and 4 lbs. in evening. The temperature was 39° and 39.3°C.

Oct. 13. Calf drank 4 lbs. of milk morning and evening. Temperature 39° and 39.4°C.

Oct. 14. Calf drank 4 lbs. in morning. Feces soft, yellow, streaked with blood. Evening food withheld.

Oct. 15. Given 4 lbs. of milk. Temperature 39.8°C. Calf had a chill. Respirations 48; pulse 100. Feces soft with fetid odor. Discharge from both nostrils, mucous in character. Temperature, 5 p.m., 40.2°C. Calf drank 2 lbs. of milk. Temperature 39.6° at 9 p.m. Calf can stand up but unable to move legs freely.

Oct. 16. Temperature 38.6–39.1°C. during the day. Calf takes 1 lb. of milk, is very weak, unable to hold up head. Feces soft, streaked with blood. Legs cold. Dies at 11 p.m. and is placed in refrigerator.

The autopsy made early next morning. Weight of calf 88 lbs. The only noteworthy feature is an intense congestion of the medullary portion of kidneys. Urine from the bladder has a specific gravity of 1.011, yellow, cloudy, alkaline, albumin 0.2 per cent Esbach; sugar absent.

Cultures from spleen, liver, and kidneys show large numbers of colonies of non-motile bacilli like those from Calf 663. They ferment dextrose and lactose but not saccharose.

Calf 669.—Male, born Oct. 18, 1921, 1.20 p.m., first calf of a native cow. Remained with dam until 5 p.m. Then taken to an isolation unit at the Depart-

ment. The dam had been vaccinated same as dam of Calf 664. Calf was not fed in the evening. It refused milk next morning. In the evening it drank 2 lbs. The milk came from Cow 601.

The calf continued normal with temperature fluctuating between 37.5° and 38.5°C. until Nov. 5, when the daily taking was discontinued.

The calf was killed Feb. 14, 1922. It weighed 225 lbs. No lesions were found.

Calf 680.—Male, born during the night of Nov. 1 to 2, 1921, first-born of a native cow. The calf, which had suckled, was removed in the morning to an isolation unit at the Department and fed milk of Cow 678.

From Nov. 2 to 4, the stools were very liquid. Some diarrhea was again noticed on Nov. 21 and 22. The temperature fluctuated between 38° and 39°C. at this time. Otherwise the calf remained well and thrifty until sold Dec. 10.

Calf 681.—Male, born Nov. 2, 1921, 2 p.m., of a native cow. Probably the third or fourth calf. Calf kept from suckling the dam and brought over to a Department unit at 4.30 p.m. Fed 2 lbs. of milk.

Nov. 3. Fed 3 lbs. of milk in the morning. Large mass of feces passed with tendency to become liquid. Discharge from nose. Temperature 38.7–39.0°C.

Nov. 4. Takes 3 lbs. of milk. Discharge from nostrils more profuse. Calf grows weaker and unable to stand later in day. Temperature 39.2–39.7°C.

Nov. 5. Calf very weak, refuses food. Is unable to get up. In this case it was thought best to kill the calf to eliminate postmortem changes. Killed by stunning and cutting vessels of neck. Weight 73 lbs. The autopsy was negative except for the following.

The fourth stomach contained a mass of coagulated milk about 8 cm. in diameter. The mucosa of this organ and of the small and large intestines normal. The liver cells contain considerable fat. The kidneys are slightly enlarged, very firm to the touch, and deeply congested throughout. Urine from the bladder is yellowish, somewhat cloudy, slightly acid, specific gravity 1.030, albumin 0.15 per cent. Sections of the kidney show a complete injection of the entire capillary system. Those of spleen show numerous hemorrhages in the parenchyma.

Cultures were made from spleen, liver, and kidneys as follows: A bit of tissue about the size of a pea or small bean was transferred to an agar slant, rubbed over the surface, and deposited in the condensation water. A platinum loop dipped into this condensation water was rubbed over the slanted surface of a second tube. The numbers of colonies in the first tubes were countless. Those from the second tubes were 3 for the spleen, 15 for the liver, and 6 or 7 for the kidney. Dilutions from an intestinal loop planted in agar yielded the same kind of colonies; namely, the saccharose-non-fermenting *B. coli.*

Calf 682.—Born Nov. 2, 1921, before 6 a.m., of an eastern cow in her third pregnancy. This calf weak at birth remained so and was unable to take food. It grew weaker and was killed when about 33 hours old, Nov. 3, 2.30 p.m., to determine the presence or absence of *B. coli* septicemia.

The autopsy showed the absence of any food in the stomachs. These still contained the fetal fluids, viscid, bile-stained. The mucosa of nearly the entire

fourth stomach and pyloric valve was sprinkled over with minute, irregularly star-shaped hemorrhages, about 1 mm. apart. No surface hemorrhage. The small intestine nearly empty, normal. The lowest 24 inches of large intestine sprinkled with punctiform hemorrhages merging into a deep universal congestion in the rectum. The liver cells contained much fat. Other organs appear normal.

The cultures from spleen, liver, and kidney showed coalescing layers of growth in those in which bits of tissue had been placed. Those inoculated with a loop of condensation water from the preceding contained 6 or 7 colonies (spleen), 1 colony (kidney), and 6 or 7 colonies (liver). Dilutions of contents of an intestinal loop spread over agar showed the same type of colony. The bacilli were of the saccharose-non-fermenting type.

Calf 685.—Male, born Nov. 9, 1921, at 2 p.m., of a native cow in her third pregnancy. Calf removed from dam immediately after birth. At 3.10 p.m. placed with a cow which had calved 5 days ago. Calf began to suckle at 3.20 p.m. At 4 p.m. it was taken to a Department unit.

Nov. 10. Calf took 3 lbs. of milk early. Passed dark greenish masses of meconium. Feces tending to become liquid during the day. Temperature rose from 38.5°C. in morning to 39.6°C. in the late afternoon. Calf refused to take but a little milk late in the day, now very weak.

Nov. 11. Calf died at 5 a.m. From the autopsy record the following may be mentioned.

The umbilical vessels show only the usual amount of hemorrhagic discoloration. Rumen empty, normal. Fourth stomach normally distended with an opalescent, watery fluid and many small casein clumps. An area about 10 cm. in diameter of the leafy portion sprinkled with surface hemorrhages. Faded deeper hemorrhages in rest of leafy portion. In the small intestine there are well defined areas of deep congestion through the entire tube. There are linear congestions on crests of longitudinal folds of large intestine. Liver cells contain considerable fat. Spleen flabby. Some papillæ of kidneys congested. Heart and lungs negative.

Cultures from spleen, liver, and kidney show the same invasion of *B. coli* as in the preceding cases.

Calf 687.—Male, born Nov. 9, 1921, at 4.15 p.m. Remained with the dam until Nov. 10, 11.10 a.m., when it was removed to the Department animal house. The dam had been brought from Michigan in October. The calf refused to take milk (from Cow 679) the first evening, but drank next morning and thereafter. The temperature fluctuated between 38° and 39°C., rarely falling as low as 37.5°C. Nothing abnormal was observed. On Dec. 10 the calf was sold to the butcher.

Calf 694.—Born Nov. 25, 1921, at 1.30 p.m. Dam an eastern cow. Not known how many calves preceded this one. There was some difficulty in getting the calf to suckle the dam. At 4 p.m. it was induced to take colostrum from two quarters for about 10 minutes. At 4.30 p.m. it was transferred to the Department units. The subsequent feeding occasioned no special difficulties.

Up to Jan. 2, 1922, the temperature maximum and minimum were 39.8° and 37.5°C. Most of the morning and afternoon readings fluctuated between 38° and 39°C.

The calf was found dead unexpectedly at 7 a.m., Jan. 2. It had not been seen since 11 p.m., Jan. 1. It was at once placed in the refrigerator until Jan. 3. The autopsy presented the following points.

The mucosa of the digestive tract was abnormally pale. The fourth stomach contained a firm milk coagulum in which were enmeshed pieces of twine eaten by the calf from the straw bedding. There was more than the usual bile staining in the small and large intestines. The heart cavities contained soft dark clots and the endocardium was deeply suffused with blood coloring matter. The spleen was very flabby and of normal size. Some large bacilli (postmortem?) present. The liver was yellowish in color. There were a moderate amount of fat in the liver cells and some orange pigment. Bacteria not seen in films. The kidneys on section showed closely set, pale, linear streaks, passing vertically through cortex. In fresh scrapings the nuclei of the convoluted tubule epithelium contained each an irregular, refringent block or concretion. The thymus within the thorax was densely studded with hemorrhages up to 2 mm. in diameter. The cervical portion was free from them. Cultures were not made.

Calf 697.—Female, born Nov. 29, 1921, 2 p.m., of a native cow in her third pregnancy. The calf was unable to stand up for a time. At 2.45 p.m. it was transferred to the Department units and gently dried by rubbing with cloths. At 4 p.m. it was allowed to suckle another cow (No. 600) but fed poorly and obtained little.

Nov. 30. Temperature 38.5° and 39°C. Took 2½ lbs. of milk morning and evening. Voids large masses of meconium. The milk came from Cow 679. Dec. 1. Temperature 39° and 39.7°C. Drank 3 lbs. milk morning and evening. Mucopurulent discharge from nostrils. Dec. 2. Temperature 39.3° and 40.2°C. Food as yesterday. Dec. 3. Temperature 40°C. Discharge from nostrils abundant, thick. Feces normal. Dec. 4. Temperature 39.3° and 39°C. Food as before. The calf soon recovered from the rhinitis and gained weight normally. It was used in another experiment and sold to the butcher in fine condition Feb. 2, 1922. The inspector reported organs normal.

Calf 698.—Male, born Dec. 6, 1921, between 12 and 1, noon, fourth calf of a native cow. The calf was allowed to take the colostrum and then removed at 2.20 p.m. to the Department buildings.

The calf refused to drink next day until evening when some milk was slowly poured into its mouth. At 11 p.m. it drank about 12 ounces from a bottle. On the following day it began to take food in larger quantities. The temperature rose to 39.6°C. on Dec. 7. Thereafter it fluctuated between 38° and 39.5°C., falling to 37.4°C., Dec. 15. Temperature taking discontinued after Dec. 23. Calf sold to butcher Feb. 2, 1922. Inspector reported the organs normal.

Calf 699.—Male, born Dec. 6, 1921, at noon, of a native cow in her third pregnancy. Calf removed at once to Department buildings and dried off by rubbing with cloths. Fed about 2 lbs. of milk from Cow 678.

Dec. 7. Calf drank about 2½ lbs. of milk morning and evening. A large amount of feces was passed during the day, gradually assuming a liquid condition. Temperature 38.2° and 39.7°C. Dec. 8. Calf died at 8 a.m. At the autopsy the following appearances were noted.

The mucosa of pharynx and larynx cyanotic. A milk curd about 3 to 3½ inches in diameter in the fourth stomach and some milky fluid. Mucosa dark bluish red. The mucosa of small intestine deeply injected throughout. Microscopic examination shows complete injection of capillary system of villi. The large intestine similarly congested with intensification in the rectum. Bowels practically empty. Thoracic organs show nothing noteworthy. Liver cells contain considerable fat. The spleen is splashed with subcapsular hemorrhages. The kidneys are deeply congested throughout. Urine taken from the bladder is clear, yellow, acid. Specific gravity 1.025. A trace of albumin present.

Cultures from spleen, kidney, and liver show as heretofore the presence of large numbers of colon bacilli, not fermenting saccharose. The same type isolated from two segments of the small intestine. Other species not in evidence.

Calf 715.—Female, born Jan. 5, 1922, about 4 a.m., of a native cow in her first pregnancy. The calf was left with dam until 4 p.m. and then brought to the Department units.

The calf was fed milk from Cow 712 the next day and feeding continued three times daily. The temperature remained normal and the appetite good. From Jan. 18 on it was taking 12 lbs. of milk. Jan. 26. *B. bovisepticus* was introduced into one nostril and the reaction following was slight. It was found dead unexpectedly Feb. 19 at 5.30 a.m. when 45 days old. The watchman reported behavior of the calf similar to that of No. 757 before death. The calf was placed in the refrigerator until Feb. 20.

The only noteworthy changes observed at the autopsy were as follows: The fourth stomach contains milk clots mixed with twine picked out of bedding. The mucosa is partly digested. Extensive subendocardial hemorrhages in the left ventricle. All cavities contain soft dark clots. The spleen is very flabby and not enlarged. The liver cells contain large fat globules. The kidney cortex is regularly marked with grayish streaks. The intrathoracic portion of the thymus is permeated with hemorrhages 1 to 2 mm. in diameter.

Cultures of bits of tissue from liver, spleen, and kidneys in plain and blood agar slants and in fermentation tubes remain indefinitely sterile. Similarly, cultures from the heart's blood.

Calf 716.—Female, born Jan. 11, 1922, 1.15 p.m., of a native cow in her third pregnancy. Calf taken from cow and removed to the Department buildings, rubbed until nearly dry. Fed 1½ lbs. of milk from Cow 712 in evening from a nursing bottle.

Jan. 12 to 16. The calf takes milk regularly and is fed three times a day. The temperature is within the normal range. Jan. 17 to 18. Temperature 39.5°C. Evidence of joint involvement in left elbow and tarsal joints. Calf constipated. Jan. 19. Calf moves more easily about stall. Is taking 9 lbs. of milk daily in three doses. Feb. 1. Slight lameness in left fore leg and some weakness in hind limbs. Feb. 11. Swelling of left tarsal joint probably a distension of synovial sheaths. Feb. 28. Temperature has been normal to date. The joint troubles have practically disappeared. This calf was killed Mar. 16, when 2 months and 5 days old. There were no abnormal appearances of the organs and the cultures made as heretofore remained sterile.

Calf 717.—Male, born Jan. 11, 1922, at 4 p.m., of a midwestern cow. First calf since coming into this herd. The calf was removed at 5 p.m. to the Department buildings and dried off by gentle friction with cloths. Fed at 5.30 p.m. from a nursing bottle about 1 lb. of milk from Cow 678.

Jan. 12 to 19. Fed three times daily about 2 lbs. of milk each time. Jan. 20. Temperature up to 40.2°C. this morning. Calf is very weak but is taking its food as usual. Constipated. Temperature 41°C. at 8 p.m. Respirations short and irregular. Jan. 21. Calf took its food three times today. Is slightly better. Umbilicus slightly swollen and tender. Jan. 22. Calf too weak to get up and take its food. Dies at 10 a.m. Refrigerated at once until next day.

Jan. 23. Autopsy. Weight of calf 92 lbs. Umbilicus has a dry black tape still attached. Base slightly thickened and indurated. When tape removed, a circular area is exposed covered with a thin layer of pus and communicating with a closed diverticulum with purulent contents. No communication with umbilical vein, which is closed. The digestive tract normal with exception of some deeply congested patches of mucosa in ileum. Lungs slightly edematous and congested. Some small fibrin masses in pericardial sac and a few hemorrhages under epicardium. Liver, spleen, and kidneys softer than normal. Slight vascular injection of tissues within tarsal joints. Urine from the bladder contains only a trace of albumin. Specific gravity 1.012.

Cultures from spleen, liver, and kidneys show presence of large numbers of *B. coli.* Many colonies of the same bacilli in pure culture obtained from the fluid in one tarsal joint.

Sections of fixed and hardened tissue presented the following data. Spleen: Foci or colonies of *B. coli*-like rods scattered regularly through the section. Liver: One minute abscess in the section. Every liver cell contains one or more (fat) vacuoles. Each vacuole contains a central coccus-like body stained reddish (fat crystals). Kidney: Fibrin thrombi in small vessels of cortex. Occasional foci of polynuclear cells, interstitial hemorrhages, and *B. coli*-like rods filling capillaries.

Calf 718.—Male, born Jan. 12, 1922, at 7.45 a.m. Dam brought from Michigan Nov. 20, 1921. To make certain that the calf had received colostrum it was placed with the dam again at 11 a.m. and allowed to suckle the four quarters, in

all for 20 minutes. It was then brought to the Department buildings. The milk fed was from Cow 678. No difficulty was experienced in starting the artificial feeding. The maximum and minimum temperatures until Feb. 2 were 38.8° and 37.8°C. On this day it was sold to the butcher. Organs were reported normal by inspector.

Calf 757.—Male, born Jan. 23, 1922, at 9 a.m., of a native cow in her third pregnancy. The calf suckled the dam about 15 minutes before removal to the Department units. The cord was treated in the same way as that of Calf 893. The calf was fed three times daily with milk of Cow 678. The temperature and appetite continued normal. On Feb. 13, being in fine condition, it was used in another experiment, *B. bovisepticus* was introduced into a nostril. The reaction was slight.

On Feb. 17, when the calf was 22 days old and had been without abnormal symptoms thus far, it suddenly (1.55 p.m.) became very excited. It moved in a circle from left to right and lowed continually. The respirations were noisy, labored, the tongue protruded slightly, and there was more or less frothy mucus about the muzzle. The calf was forcibly restrained by being thrown and held. After a time the convulsive movements became less pronounced and the dyspnea more marked. It soon was unable to rise after being released and died 15 minutes after it had been discovered in this condition. This calf up to the time of the seizure had been in excellent condition and had even taken its milk early on the day of death as usual.

The autopsy showed nothing abnormal, except numerous small hemorrhages through the intrathoracic portion of the thymus and some minute hemorrhages under epicardium. The fourth stomach contained pieces of twine in the milk coagulum.

Cultures on plain and blood agar slants with bits of tissue from spleen, liver, and kidneys and with heart's blood, sealed, remained free from growth after 5 days incubation.

Calf 759.—Male, born of a native cow in her second pregnancy, Jan. 25, 1922, 3.20 p.m. Removed from the dam before suckling and taken to the Department buildings where it was dried off by gentle rubbing with cloths. The umbilical cord had been torn off close to the skin. The exposed area was washed, with 0.1 per cent mercuric bichloride and dusted with powdered boric acid. The Calf was very weak and scarcely able to stand up. Fed from bottle ¾ lb. of milk from Cow 678. Fed again 10.35 p.m.

Jan. 26. Fed three times today. Dry meconium being passed. Calf stronger Jan. 27. Temperature 39°C. Takes food regularly. Soft yellow feces passed in large amounts, becoming liquid. Jan. 28. Temperature 39.1–39.2°C. Feces liquid. Jan. 29. Temperature 39–38.4°C. Lameness appears in right carpal and left tarsal joints. Calf quite weak. Feb. 4. Temperature has ranged close to 39°C. Some swelling of right carpal joint and stiffness in hind limbs.

Feb. 21. Calf has been improving and taking about 12 lbs. of milk daily. Killed today by stunning and cutting vessels of neck. Weight 87 lbs.

The autopsy showed the following abnormalities. In the right cephalic lobe of lungs some collapsed areas, each 4 to 5 mm. in diameter. Both kidneys spotted with white patches varying from mere specks to areas of a square centimeter. These areas correspond to the bases of solid foci reaching to medulla. A vertical section through these foci is either linear, square, fan-shaped, or oval. The involved tissue is white, smooth, glistening on section and quite firm. The distribution is irregular, not associated with any structures in the cortex. The urine taken from bladder is clear amber-colored, alkaline, specific gravity 1.020. A trace of albumin is present. The liver shows a distinct fatty zone on the periphery of the lobules.

In the right radiocarpal articulation a small quantity of an opaque fluid containing soft mucoid flakes together with a firm whitish fibrinous mold of a portion of the joint cavity, loose, and easily removed with forceps. The exudation and edema extend from the joint up the external aspect of the leg (radius) between the muscles for 3 to 4 inches. The joint exudate consists of polynuclear leucocytes.

Cultures were made with bits of tissue from spleen, liver, kidney, and the diseased joint. The spleen culture remained sterile. In the liver and kidney tubes several species of bacteria appeared, indicating a miscellaneous infection of the body. The joint cultures contained *B. coli.*

Calf 893.—Male, born Feb. 9, 1922, about 6 a.m. First-born of a native cow. Calf at first removed from dam as not having suckled, but owing to uncertainty in this respect, it was allowed to suckle dam at 11 a.m. for 20 minutes and then taken to the Department buildings. The umbilical cord was snipped off, leaving about 1 inch. This was dipped in 0.1 per cent mercuric chloride and covered with powdered boric acid. First meal taken the same evening was milk from Cow 712. There was no difficulty in feeding. The temperature was taken twice daily until March when the calf was used in another experiment. During this time, the maximum and the minimum temperatures were 39.4° and 37.7°C. The calf was killed Mar. 28. The only abnormality found was pigmentation of the mucosa of cecum, along the course of the larger vessels.

Calf 894.—Male, born Feb. 9, 1922, 6.30 a.m., of a native cow in her first pregnancy. The calf was removed from the dam but was not taken to the Department buildings until about noon. It was probably exposed and chilled in this interval. It was then gently rubbed with cloths and the umbilical cord treated as in earlier cases. Fed at noon and night from a bottle with milk from Cow 712. Feb. 10. Fed three times today. Temperature 38.8° and 39.2°C. Large amounts of dark greenish feces passed today. Feb. 11 to 12. Temperature range 38.8–39.4°. Tendency towards diarrhea. Feb. 13. Calf is very weak. Takes milk twice, but unable towards night to stand. Feces fluid. Dies at 10.30 p.m. Placed in refrigerator until next morning. Weight 73 lbs.

Autopsy negative except for petechiæ in fourth stomach and a deep congestion of the mucosa of ileum. Tubes inoculated from spleen, liver, and kidneys show large numbers of colonies of *B. coli*.

Calf 895.—Male, born about 7 a.m., Feb. 10, 1922, of an eastern cow in her fourth pregnancy. Removed promptly from the dam and taken to the Department units at 10.20 a.m. where it was dried and umbilicus treated. The calf was fed at noon and evening with milk from Cow 678.

Feb. 11. Large amounts of relatively firm meconium discharged last night. Fed three times today. Temperature 38.8–39.7°C. Feb. 12. Fed as before. Calf growing weak and unable to stand later in day. Temperature 39.1–39.5°C. Feb. 13. Calf unable to stand up and drink. Nostrils filled with mucus. Temperature falling, 38.4–37.1°C. Died at 11 p.m. and refrigerated soon after.

Feb. 14. Autopsy presents the following points. The umbilical vein just beneath abdominal wall is distended with a normal clot about 2 cm. in diameter. No communication with exterior. Fourth stomach contains some curds and milky fluid. Many superficial hemorrhagic points in leafy portion. Rectum deeply congested. Some dried yellowish fecal matter in it, blood-stained. Both carpal joints contain several cubic centimeters of a turbid fluid with flakes made up of polynuclear leucocytes.

Interarticular surfaces of tibiotarsal joints deeply congested. Cultures from spleen, liver, and kidneys show coalescent layers of growth. Cultures from metatarsal joints, large numbers of isolated colonies. These and the growth from spleen were replated and found to be the same type of *B. coli*.

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THE RELATION BETWEEN AGE AND THE CONCENTRATIONS OF PROTEIN FRACTIONS IN THE BLOOD OF THE CALF AND COW.

BY PAUL E. HOWE.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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The marked variations in the quantities of certain protein fractions present in the blood of the new-born calf (1, 2) and the differences between the blood of young calves and of the adult cow led us to study the changes which occur in bovine blood with increasing age. For this purpose we have studied the blood of three calves from birth to an age of approximately 2 to 3 months. Samples of blood have been taken from other animals; three heifers 6 months old, three heifers 12 months old, fifteen virgin heifers 17 to 22 months old, and fourteen pregnant heifers $2\frac{1}{2}$ years old. The heifers 17 to 22 months old were about to be bred and those 30 months old had been pregnant about 3 months. These observations are supplemented by analyses of the blood of other animals for a short period (2) and data obtained in studies in another connection.

Of the calves which were studied continuously one (Calf 669) received colostrum of a high protein content, a second (Calf 889) received colostrum which was comparatively poor in globulin, and a third (Calf 898) did not receive colostrum but was fed ordinary whole milk from birth. Blood was collected by needle from the jugular vein and when plasma was obtained coagulation was prevented by sodium citrate. Blood samples were taken 1 hour after feeding. The calves were fed ordinary whole milk for 1 month after which they were given small amounts of grain and hay.

Determinations were made of total nitrogen, fibrin, or fibrinogen nitrogen, nitrogen content of the protein precipitated by concentrations of sodium sulfate of 14.2, 17.4, and 21.5 per cent, and of the

non-protein nitrogen. From these determinations values were calculated for fibrin, euglobulin, pseudoglobulins I and II, and albumin according to the procedures previously outlined (3). The data are contained in Tables I to VI and Chart 1. The relation between the

TABLE I.

*Data Relating to the Variations in the Blood Proteins of Calf 898 Which Did Not Receive Colostrum soon after Birth.**

Age.	Total N.	"Serum" N.	Fibrin N.	Euglobulin N.	Pseudoglobulin I N.	Pseudoglobulin II N.	Total globulin exclusive of fibrin.	Albumin N.	Non-protein N.
New-born.	0.636	0.581	0.055	0.016	0.028	0.144	0.189	0.322	(0.070)
days									
1	0.659	0.602	0.057	0.008	0.050	0.144	0.202	0.330	(0.070)
2	0.703	0.604	0.099	0.002	0.052	0.139	0.183	0.351	(0.070)
3	0.758	0.636	0.122	0.000	0.046	0.159	0.205	0.361	(0.070)
4	0.757	0.594	0.163	0.045	0.025	0.120	0.190	0.359	0.045
6	0.862	0.656	0.206	0.047	0.046	0.157	0.240	0.379	0.037
8	0.901	0.668	0.233	0.008	0.060	0.126	0.194	0.437	0.037
14	0.957	0.763	0.194	0.023	0.051	0.124	0.198	0.511	0.054
20	0.887	0.759	0.138	0.029	0.012	0.153	0.194	0.511	0.054
27	0.854	0.740	0.114	0.037	0.049	0.107	0.183	0.516	0.041
34	1.024	0.804	0.220	0.048	0.069	0.149	0.260	0.503	0.041
41	0.977	0.876	0.101	0.035	0.120	0.111	0.266	0.560	0.050
48	0.974	0.944	0.030	0.095	0.101	0.113	0.309	0.577	0.058
55	0.961	0.887	0.074	0.037	0.132	0.103	0.272	0.570	0.045
62	0.998	0.935	0.063	0.072	0.113	0.165	0.350	0.541	0.045
69	0.895	0.841	0.054	0.049	0.095	0.099	0.243	0.553	0.045
76	0.891	0.847	0.054	0.051	0.099	0.074	0.224	0.590	0.033
83	0.875	0.828	0.047	0.052	0.104	0.084	0.240	0.548	0.040
90	0.918	0.841	0.077	0.063	0.100	0.088	0.261	0.544	0.036
97	0.959	0.862	0.097	0.066	0.088	0.136	0.290	0.532	0.040
111	0.981	0.908	0.073	0.049	0.114	0.124	0.287	0.573	0.048

* Results are expressed as grams of nitrogen in 100 cc. of blood plasma or serum.

corpuscles and plasma was determined by measuring the volume of each after centrifuging at a constant speed. The average proportions of corpuscles and plasma for Calves 898 and 899 were 38 to 62 and 44 to 55, respectively. These relations held throughout the experiment.

In the chart the data from each of the calves (Calves 669,[1] 899, 898), have been plotted for each of the blood constituents estimated with the exception of the total plasma nitrogen and the non-protein nitrogen. In place of the total plasma nitrogen, values for serum

TABLE II.

*Data Relating to the Variations in the Blood Proteins of Calf 899 Which Received Colostrum Relatively Low in Globulin.**

Age.	Total N.	"Serum" N.	Fibrin N.	Euglobulin N.	Pseudoglobulin I N.	Pseudoglobulin II N.	Total globulin exclusive of fibrin.	Albumin N.	Non-protein N.
Colostrum.	1.916	Casein = 1.122		0.258	0.181	0.095		0.101	0.062
New-born.	0.685	0.623	0.062	0.033		0.186	0.219	0.363	0.041
days									
1	0.877	0.764	0.093	0.116	0.204	0.136	0.451	0.276	0.038
2	0.949	0.808	0.141	0.128	0.151	0.145	0.424	0.335	0.049
3	0.950	0.853	0.097	0.115	0.185	0.174	0.474	0.334	0.045
4	1.006	0.866	0.140	0.132	0.198	0.144	0.474	0.347	0.048
5	0.848	0.726	0.122	0.073	0.146	0.166	0.375	0.306	0.045
8	0.927	0.792	0.135	0.083	0.144	0.132	0.359	0.392	0.041
12	0.935	0.852	0.083	0.136	0.141	0.152	0.429	0.367	0.054
18	0.878	0.794	0.084	0.072	0.124	0.103	0.299	0.450	0.045
25	0.901	0.833	0.068	0.078	0.093	0.140	0.311	0.483	0.041
32	0.947	0.883	0.064	0.095	0.083	0.165	0.343	0.495	0.045
39	0.882	0.824	0.058	0.043	0.083	0.100	0.226	0.548	0.050
46	0.955	0.899	0.056	0.072	0.087	0.142	0.301	0.540	0.058
53	0.864	0.827	0.037	0.054	0.100	0.133	0.287	0.482	0.058
60	0.923	0.842	0.081	0.075	0.093	0.097	0.265	0.532	0.045
67	0.856	0.784	0.072	0.050	0.087	0.127	0.264	0.475	0.045
68	0.858	0.796	0.062	0.083	0.084	0.104	0.272	0.487	0.037

* Results are expressed as grams of nitrogen in 100 cc. of blood plasma or serum.

nitrogen obtained from the analysis of the filtrates from the precipitation of fibrin or fibrinogen have been recorded. These results were taken for comparison because the fibrinogen of blood appears to vary somewhat independently of the other blood constituents.

[1] Data relating to the absorption and disappearance of agglutinins of Calf 669 have been presented in another connection (4).

From an inspection of the tables and of the chart the following points are evident:

Serum Nitrogen.—During the first weeks of life the quantity of serum nitrogen present in the blood of young calves depends upon the

TABLE III.

*Data Relating to the Variations in the Blood Proteins of Calf 669 Which Received Colostrum Relatively High in Globulin.**

Age.	Total N.	"Serum" N.	Fibrin N.	Euglobulin N.	Pseudoglobulin I N.	Pseudoglobulin II N.	Total globulin exclusive of fibrin.	Albumin N.	Non-protein N.
New-born.		0.768		0.039	0.051	0.175	0.265	0.422	(0.081)
2 hrs. 40 mins.		0.768		0.000	0.128	0.177	0.282	0.405	(0.081)
5 " 40 "		0.960		0.133	0.264	0.162	0.559	0.319	0.081
19 " 40 "		1.126		0.307	0.333	0.158	0.798	0.290	0.038
days									
3		1.160		0.298	0.320	0.133	0.751	0.358	0.051
13		1.186		0.299	0.141	0.111	0.551	0.558	0.077
21		1.071		0.239	0.128	0.073	0.440	0.597	0.034
31		0.972		0.064	0.174	0.069	0.307	0.622	0.043
41		1.005		0.045	0.196	0.071	0.312	0.667	0.026
50		0.994		0.062	0.115	0.115	0.294	0.666	0.034
57		0.959		0.051	0.098	0.102	0.251	0.661	0.047
65		0.937		0.033	0.189	0.077	0.289	0.601	0.047
71		0.948		0.082	0.120	0.098	0.289	0.616	0.043
78		0.917		0.030	0.106	0.107	0.233	0.653	0.021
85		0.937		0.042	0.058	0.087	0.187	0.713	0.037
92		0.931		0.036	0.111	0.085	0.232	0.658	0.041
106		1.032		0.071	0.093	0.173	0.337	0.646	0.049
113		0.959		0.072	0.091	0.153	0.319	0.584	0.049
118		1.038		0.081	0.114	0.113	0.308	0.681	0.049

* Results are expressed as grams of nitrogen in 100 cc. of blood plasma or serum.

quantitative nature of the diet just after birth. If the calf does not receive colostrum but is fed ordinary milk the serum nitrogen increases gradually for about 6 weeks. This increase is due, essentially, to the increase in albumin. In case colostrum has been ingested the serum nitrogen values indicate the effect of the absorption of globulins

TABLE IV.

Data Relating to the Proteins in the Blood of Calves 6 and 12 Months Old.

Age.	Total N.	"Serum" N.	Fibrin N.	Euglobulin N.	Pseudoglobulin I N.	Pseudoglobulin II N.	Total globulin exclusive of fibrin.	Albumin N.	Non-protein N.
mos.									
6	1.098	1.027	0.071	0.124	0.210	0.132	0.466	0.516	0.045
	1.082	0.991	0.091	0.104	0.223	0.120	0.447	0.494	0.050
	1.030	1.002	0.028	0.129	0.178	0.140	0.437	0.507	0.058
Average.....	1.070	1.007	0.063	0.116	0.203	0.131	0.450	0.506	0.051
12	1.087	1.038	0.049	0.077	0.206	0.136	0.419	0.547	0.062
	1.074	1.006	0.068	0.070	0.210	0.145	0.425	0.523	0.058
	1.059	1.021	0.028	0.097	0.210	0.161	0.468	0.503	0.050
Average.....	1.073	1.022	0.048	0.081	0.208	0.137	0.437	0.524	0.057

TABLE V.

*Data Relating to the Proteins in the Blood of Different Non-Pregnant Heifers 18 to 22 Months Old.**

Age.	Total N.	"Serum" N.	Fibrin N.	Euglobulin N.	Pseudoglobulin I N.	Pseudoglobulin II N.	Total globulin exclusive of fibrin.	Albumin N.	Non-protein N.
mos.									
17	1.132	1.027	0.105	0.083	0.202	0.156	0.441	0.516	0.070
18	1.219	1.043	0.176	0.115	0.260	0.120	0.495	0.478	0.070
18	1.359	1.270	0.089	0.181	0.355	0.126	0.672	0.520	0.078
18	1.104	1.002	0.102	0.049	0.256	0.103	0.408	0.524	0.070
18	1.060	0.978	0.082	0.034	0.235	0.091	0.363	0.545	0.070
19	1.185	1.130	0.055	0.070	0.256	0.152	0.478	0.582	0.070
19	1.082	1.002	0.080	0.062	0.231	0.136	0.429	0.515	0.058
20	1.099	1.047	0.052	0.090	0.239	0.145	0.474	0.515	0.058
20	1.305	1.126	0.179	0.042	0.395	0.157	0.594	0.462	0.070
20	1.261	1.134	0.127	0.057	0.380	0.132	0.569	0.511	0.054
20	1.316	1.114	0.202	0.042	0.375	0.140	0.557	0.499	0.058
21	1.175	1.035	0.140	0.078	0.279	0.138	0.495	0.490	0.050
22	1.168	1.097	0.071	0.035	0.353	0.132	0.520	0.515	0.062
22	1.183	1.048	0.135	0.058	0.301	0.136	0.495	0.483	0.070
22	1.184	1.084	0.100	0.036	0.293	0.136	0.464	0.557	0.062
Average.....	1.188	1.073	0.115	0.069	0.296	0.133	0.494	0.514	0.064

* Results are expressed as grams of nitrogen in 100 cc. of blood plasma or serum.

from the colostrum. After approximately 6 weeks serum nitrogen fluctuates somewhat but is lower than the concentration found in the adult animal or in the calves 6 months old. The adult values appear to be attained between 3 and 6 months of age.

Albumin Nitrogen.—Changes in the albumin nitrogen are more or less independent of the variations in the other proteins. The value at birth is slightly higher than on the following day. From this time

TABLE VI.

Data Relating to the Proteins in the Blood of Different Pregnant Heifers 30 Months Old.

Age.	Total N.	"Serum" N.	Fibrin N.	Euglobulin N.	Pseudoglobulin I N.	Pseudoglobulin II N	Total globulin exclusive of fibrin.	Albumin N.	Non-protein N.
mos.									
	1.079	1.015	0.064	0.099	0.260	0.112	0.471	0.482	0.062
	1.052	0.964	0.088	0.061	0.214	0.136	0.411	0.491	0.062
	1.142	1.083	0.059	0.072	0.293	0.128	0.493	0.528	0.062
	1.043	0.957	0.086	0.116	0.251	0.136	0.503	0.396	0.058
	1.129	1.070	0.059	0.101	0.268	0.120	0.489	0.523	0.058
	1.085	1.033	0.052	0.072	0.297	0.148	0.517	0.458	0.058
30	1.064	1.010	0.054	0.129	0.198	0.099	0.416	0.532	0.062
	1.095	1.047	0.048	0.086	0.243	0.120	0.449	0.540	0.058
	1.225	1.136	0.089	0.092	0.364	0.140	0.596	0.478	0.062
	1.123	1.085	0.038	0.194	0.256	0.132	0.582	0.441	0.062
	1.133	1.074	0.059	0.158	0.207	0.144	0.509	0.499	0.066
	1.090	1.021	0.069	0.130	0.177	0.161	0.468	0.487	0.066
	1.150	1.070	0.080	0.196	0.288	0.096	0.580	0.423	0.066
	1.163	1.113	0.050	0.189	0.285	0.136	0.610	0.437	(0.066)
Average.....	1.112	1.048	0.064	0.121	0.252	0.132	0.506	0.479	0.062

there is a gradual increase up to approximately 3 weeks, after which there is a tendency toward a slight increase with irregular fluctuations. The important fact to be brought out with regard to the albumin nitrogen is that changes in the concentration of albumin, particularly in the first 3 weeks of life, do not appear to be correlated with the changes in the concentration of the globulins.

Fibrin Nitrogen.—The data on fibrin nitrogen are in part, in the case of Calves 898 and 899 and of the older animals, based on duplicate

determinations by two different procedures; coagulation following recalcification with calcium chloride and precipitation with 10.6 per cent sodium sulfate. The first procedure represents fibrin and the second fibrinogen. With the quantities of plasma used the results by the two methods usually agree.[2]

From the data on Calves 898 and 899 it might appear that there is a higher fibrinogen content of the blood early in life, during the period when adjustments in the other proteins are taking place. The determinations of fibrin on various young animals indicate a considerable individual variation and no relation of age to the fibrinogen concentration of the blood. A consideration of data on the adult animals also tends to substantiate this idea. It is evident that the variations in the concentration of fibrinogen of the calves is not directly related to the variations in the other serum proteins. The daily records on Calves 898 and 899 do not lend much assistance in interpreting the variations in the fibrinogen content of the blood. Calf 898 had a temperature approximately 1° higher than Calf 899 at the time the fibrinogen content of its blood was rising but during the period of high fibrinogen values the temperature was approximately that of Calf 899. From the work of Smith and Little (5) on the effect of colostrum upon the new-born animal, it is probable that this calf was conducting a battle against an infection which was not present in the case of Calf 899. The large increase in fibrinogen at the age of 34 days in the case of Calf 898 occurred at the same time as an abraised and swollen knee-joint which was not present the period before this one and had disappeared before the time of the next analysis. Calf 899 does not present any marked temperature changes. In the latter part of the experiment he was subject to a deranged digestive apparatus due to the ingestion of binding twine in his bedding which caused partial occlusion of the rumen and interfered with his normal metabolic activities. The data obtained during the last month of life for this calf are open to question as far as it may be considered as representing a normal animal.

The work of Foster and Whipple (6) indicates that cell injury, inflammation, intoxication, or liver injury, will affect the formation of fibrin. If we admit the probable systemic infection (5), then the

[2] Unpublished data.

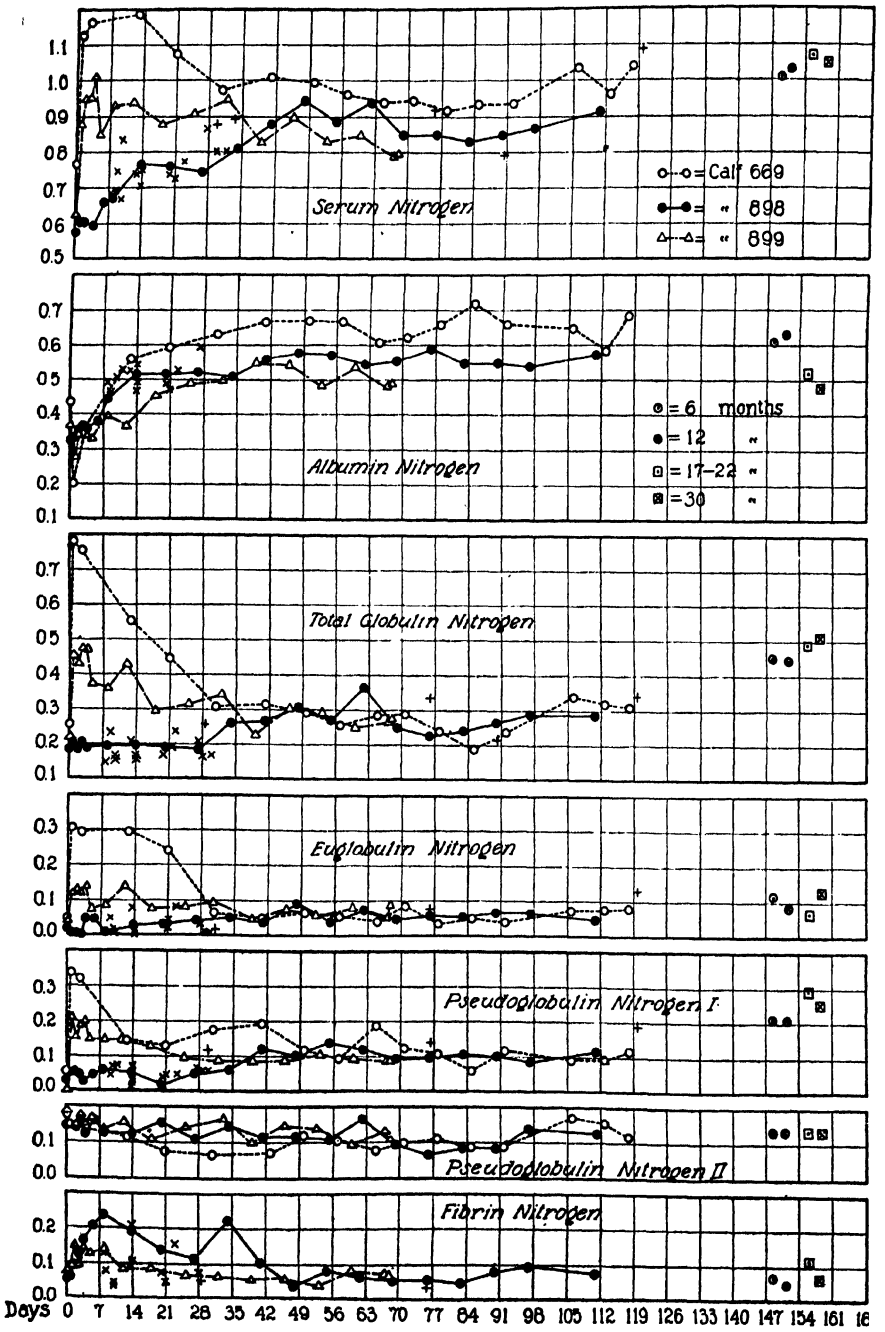
Serum Nitrogen
o-o = Calf 669
●-● = " 898
△-△ = " 899
Albumin Nitrogen
⊙ = 6 months
● = 12 "
⊡ = 17–22 "
⊠ = 30 "
Total Globulin Nitrogen
Euglobulin Nitrogen
Pseudoglobulin Nitrogen I
Pseudoglobulin Nitrogen II
Fibrin Nitrogen
Days 0 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112 119 126 133 140 147 154 161 16

CHART 1.

CHART 1. Curves showing the variations in various protein fractions of blood with increasing age. Calf 898 did not receive colostrum, Calf 899 received colostrum relatively low in globulins, and Calf 669 received colostrum rich in globulins. The crosses (×) indicate results obtained on calves which did not receive colostrum; some of these animals were injected with cow serum, some were injected and fed cow serum, and some were fed cow serum (5). The results on these animals agree essentially with those obtained on animals which had not received colostrum or which would have received milk containing very small amounts of globulins. The plus signs (+) indicate results on animals which had received colostrum soon after birth. Average data for calves 6 and 12 months of age, non-pregnant heifers 17 to 22 months old, and pregnant heifers 30 months old are plotted as indicated in the legend. The data are presented in terms of grams of nitrogen in 100 cc. of blood plasma or serum.

general high fibrin values for Calf 898 and the increase in fibrin at the time of the swollen knee-joint might be explained by the findings of these investigators.

Total Globulin.—In the case of Calf 898 which did not receive colostrum the values for total globulin nitrogen remain practically constant for 4 weeks after which they increase to values which are essentially the same as those for calves which received colostrum. At about 10 weeks there appears to be a tendency for the total globulin to decrease slightly. At 6 months the total globulin values approach those of the adult animal. The two calves which received colostrum showed a marked increase in globulin during the first day of life after which the total globulin shows a tendency to decrease during a period of from 3 to 4 weeks. Following this change the amount of total globulins is approximately the same regardless of the previous diet. The concentration of globulin present during the first 3 to 4 weeks of life is directly related to the quantity of globulin absorbed during the first days of life. Colostrum ingested by Calf 669 was very thick and rich in globulin whereas the colostrum ingested by Calf 899 was relatively poor in globulin.

Euglobulin.—Remarks with regard to the ingestion of colostrum and the variation in protein nitrogen relating to the total globulin apply to euglobulin. At birth there is essentially no euglobulin present. When euglobulin is not obtained from the colostrum or otherwise it appears in the blood gradually and at approximately 5 to 6 weeks reaches the values found in calves which received colostrum. The euglobulin content of the adult animal is variable, due to influences which have not been determined. The data relating to euglobulin in the case of Calf 898 show a certain quantity of euglobulin during the first week of life. These values are probably only in a degree accurate and are due in part to technical error. The reason for this assumption is, that in the case of serum at such ages there is not a visible precipitation at 14.2 per cent of sodium sulfate.

Pseudoglobulin I.—In the first weeks of life the pseudoglobulin I content of the blood is related to the nature of the diet immediately after birth. At an age of approximately 5 to 6 weeks the values are the same no matter what the diet may have been. The adult animal has a much higher pseudoglobulin I content than the calf 3 months old.

Pseudoglobulin II.—The quantity of protein precipitated, between 17.4 and 21.5 per cent of sodium sulfate, appears to be independent of the diet and is practically constant for all ages. This condition exists in spite of considerable fluctuations in the protein content of the blood on either side of this fraction.

Animals 6 to 30 Months of Age.—Data relating to the concentration of the various protein fractions in animals 6 to 30 months of age are contained in Tables IV to VI. The average values are indicated in Chart 1. The blood samples for calves 6 and 12 months old were collected at the same time and under similar feeding conditions. The samples from the virgin heifers and the pregnant heifers were collected about 2 weeks apart. The general conditions of stabling and of feeding were essentially the same; the animals were still on winter feed and had not been pastured. The results of the analyses indicate certain minor individual variations in the distribution of the proteins of the blood. The effect of age is not particularly apparent after the calves are 6 months old. The only indication of a difference between the two groups of adult animals is in the euglobulin fraction which is higher in the non-pregnant heifers than in the pregnant heifers. On the other hand, the concentrations of fibrin are in general higher in the virgin heifers than in the pregnant heifers. This is rather surprising since, from the work of Fahræus (7) on human serum there is a greater suspension stability in pregnant than in non-pregnant women. The suspension stability appears to be in part at least related to the fibrinogen and globulin fractions of the plasma, in which fibrinogen has a greater individual effect than the other globulins. Our observations do not necessarily contradict those of Fahræus for we are dealing with a different organism and we have not made determinations of suspension stability.

A calculation of the relative proportions of globulin nitrogen and albumin nitrogen to the total nitrogen gives for virgin heifers 49 per cent of the total serum protein as globulin and 51 per cent as albumin; for the pregnant heifers the values are 51 per cent of total globulin nitrogen and 49 per cent of albumin nitrogen. These values differ from those of Robertson (8, 9) who found 36 per cent of total globulins and 64 per cent of total albumins for the ox. Robertson's average values for Hammarsten's (10) determinations of ox serum are 58 per

cent total globulin and 42 per cent total albumin. We have found a number of cases in which the serum of the adult animal contained a preponderance of globulin over the albumin but only a few animals, except in the case of calves, in which the albumins predominated over the globulins. We are dealing, of course, with the cow while Robertson may have been studying the steer; we do not have any evidence relating to sex.

DISCUSSION.

Studies of the variations in the distribution of proteins with increasing age are comparatively few. The most detailed investigations are those of C. E. Wells (11) on the rabbit and of Toyama (12) on the albino rat. Toyama's investigation followed that of Hatai (13) on the total protein content of the serum of the same animal. Reiss (14) and Utheim (15) have made observations on infants. These investigators have all determined the proteins by means of the refractometer. In the work on rabbits and rats the procedure of Robertson (9) for the separation of proteins has been used. Lewis and H. G. Wells (16) have recently presented some analyses of human blood using the method employed in this work.

It is necessary to be very cautious in comparing results obtained upon different species of animals. The work of Robertson (9) has brought out species, as well as individual, differences in the proportions of albumin and globulin in the serum of the rat, rabbit, horse, and ox. A difference between the infant and the calf is shown in the data of Lewis and Wells. These investigators confirm to a certain extent for the infant our observations on calves; they found that blood obtained from the umbilical cord of infants does not contain euglobulin. On the other hand, their data indicate quantities of pseudoglobulin I in the blood of infants comparable to those present in adult man, whereas in calves this protein is essentially absent.

The most extended series of analyses of the total protein of the blood of infants and children has been made by Utheim (15) who confirms the work of Reiss. It was found that the concentration of total protein remains practically constant at 6 to 6.5 per cent from birth to about the 10th to 11th month when it begins to rise. The adult level is reached at about the 15th month. The serum of

premature infants contains less protein, 4.5 per cent, than that of infants born at full term. The normal level for infants is not attained until about 3 months of age.

Alder (17) has studied the blood of man by means of the refractometer and viscosimeter and finds practically no difference between men and women; little difference in the composition of blood serum (*a*) between the ages of 7 and 70 years, (*b*) as a result of the ingestion of food, (*c*) following muscular activity, (*d*) between venous and capillary blood, and (*e*) from day to day. Placental blood contained, as a rule, less protein and a higher proportion of albumin than adult blood. The percentage of total protein in placental blood, 5.7 to 7.0 per cent, is higher than that for new-born calves, 3.6 to 5.6 per cent. Most of the values for calves lie between 3.6 and 4.8 per cent; only two samples out of twenty-eight showed a value above 4.8 per cent, the average is 4.4 per cent. The results of Lewis and Wells on placental blood, 4.3 to 6.7 per cent (N $\times$ 6.25), agree in general with those of Alder.

From the consideration of published data it is apparent that the blood of a new-born animal has a lower total protein content than that of the adult animal and that during the early part of life there is an increase in the total protein concentration of the serum. In infants and rats the protein content of the serum the first days after birth appears to be slightly lower than at birth or a few days later. With rats (13) there is a rapid increase in protein up to the time of weaning at which time the protein content shows some irregularity. The increase then continues until sexual maturity is attained when there is again an irregularity followed by further slight increases to the adult level. In the case of rabbits (11) and infants (13, 14) the available data indicate a gradual increase in total protein from birth to maturity.

In the studies just reviewed the effect of the nature of the diet immediately following birth has not been considered. The data presented in this paper indicate that the quantity of total protein present in the serum of calves is definitely related to the quantitative composition of the colostrum or milk ingested soon after birth. The *quantitative* variations in the composition of blood plasma imposed by the absorption of the proteins of colostrum are transient. At the

age of from 4 to 6 weeks, the quantitative effect of the absorbed protein has practically disappeared and the composition in the blood serum tends to become the same no matter what the previous diet may have been. These remarks apply to the normal animal.

The relative distribution of the various protein fractions of blood serum with increasing age has been studied extensively in but two cases, Wells on rabbits and Toyama on white rats. Wells did not find any "correspondence between the ages of the animals and the variations of the relative proportions of 'insoluble' globulin, 'soluble' globulin and albumins" in the blood serum of the rabbit. His youngest animal was 21 days old. The data of Toyama relate to the rat from birth to maturity. He found a gradual increase in quantity of globulin and albumin present in the blood serum, which was most rapid in the suckling period, 23 days. Immediately following the suckling period, at 30 days, there was a fall in the quantity of globulin but not in the albumin, while at the next age studied, 50 days, the albumin values dropped slightly while the globulin showed an increase.

The data presented on calves and cows indicate that during approximately 4 to 6 weeks of life the proportions of the different protein fractions precipitated from the blood by sodium sulfate are affected by the diet of the calf soon after birth; *i.e.*, by the character of the milk ingested. Following this period the absolute and relative proportions of globulins are approximately the same. The proportions of globulins characteristic of the adult animal are attained at the age of from 18 to 22 months. When colostrum containing euglobulin and pseudoglobulin I is fed there is a rapid absorption of protein by the calf (1). The absorbed globulins then gradually disappear, in part at least. When globulins are not absorbed they are formed gradually and attain values similar to those which occur following the ingestion of colostrum at about the time when the absorbed globulins have fallen to the average values for a calf 4 to 6 weeks old. The variations in globulin content just discussed relate particularly to those globulins precipitated by concentrations of sodium sulfate less than 17.4 per cent, euglobulin and pseudoglobulin I. Pseudoglobulin II remains relatively constant at all times. This constancy of the pseudoglobulin II fraction suggests that the variations in the other proteins with

feeding and age are true variations in protein and not the secondary effect of changes in the water content of the blood; *i.e.*, that the water content is adjusted to changes in the protein content of the blood.

The albumin concentration of calf serum is low at birth and rises rather rapidly during the first 2 weeks, at the end of which time the adult level is approached. Variations in the concentrations of albumin are apparently not affected by the ingestion of colostrum; essentially the same conditions exist whether or not globulin is absorbed. The non-protein nitrogen appears to be higher at birth, then to decrease slightly, and to rise again to the adult value, which approaches that at birth.

The proportions of albumin and globulin in non-pregnant and pregnant heifers are approximately equal. This statement applies to separations made with sodium sulfate. We have reason to believe that similar results would be obtained with ammonium sulfate and possibly a slightly higher proportion of globulin would be indicated when magnesium sulfate is used as the total globulin precipitant. The most marked difference between the adult blood and that of a calf 3 months old is in the proportion of pseudoglobulin I present in the blood. It appears that one of the chief adjustments between these ages is in the pseudoglobulin I fraction. The data which we have presented as indicating the composition of adult blood apply to females which have just reached maturity. Certain analyses indicate that the blood of older animals may vary widely, particularly in the fibrinogen, euglobulin, and pseudoglobulin I fractions. Some animals have a relatively high euglobulin concentration.

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A METHOD FOR THE SEPARATION OF SPORE-BEARING ANAEROBES FROM OTHER SPORE-BEARING BACTERIA.

BY J. HOWARD BROWN, PH.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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In the isolation of anaerobic spore-bearing bacteria the strictly aerobic bacteria are easily disposed of because they fail to grow under strictly anaerobic conditions. The heating of mixed cultures or of a suspension of milk or feces for twenty minutes at 80°C. in sealed tubes is the common method of separating sporulating from non-sporulating organisms. If carried out carefully this method serves to kill the vegetative bacteria leaving only the spores to germinate when transplanted to suitable media. In our experience the separation of the anaerobes from the facultatively anaerobic spore-bearing bacteria was attended with considerable difficulty until the method to be described was employed. Many of the facultative anaerobes grow well under anaerobic conditions. Many of them are surface spreaders on the blood agar plate. The fact that under anaerobic conditions they may not present the same typical appearance that they do under aerobic conditions makes their recognition so much the more difficult. There may be nothing about their colonies to distinguish them from anaerobes growing in the same plate. We have frequently fished numerous colonies from an anaerobic blood agar plate and found them all to be facultatively anaerobic spore-bearing bacteria.

Various means of overcoming these difficulties have been tried. Preliminary incubation of mixtures under aerobic conditions to effect the germination of the spores of aerobes, followed by exposure to heat to kill the vegetative forms was attended by some success. However, much better results were obtained by incubation of the material in fluid media under strictly anaerobic conditions for several days, then heating the mixed culture for twenty minutes at 80°C. It was found

that under strictly anaerobic conditions the facultative anaerobes did not produce spores, or at least none were found within the period of incubation of one week. Incubation must be long enough to permit the anaerobes to produce their spores. Florence (1922) has found that even under partially anaerobic conditions spore formation by a variety of aerobes and facultative anaerobes is greatly delayed. She says, furthermore, that "if transfers were made from cultures containing spores, the majority of spores carried over did not germinate in the sealed tubes." We have not studied this phase of the problem, nor do we wish to state that no spores are formed by facultative anaerobes under anaerobic conditions of growth. However, if certain of such spores do survive the process of anaerobic cultivation and the subsequent heating they are not present in sufficient numbers to cause serious trouble in the isolation of the anaerobes. After the vegetative forms have been killed by heat the mixture may be plated anaerobically at once or after germination of the spores in fluid media. For the preliminary anaerobic culture various selective media may be used to enhance the growth of various groups of anaerobic bacteria. For obtaining the growth of a large number of anaerobes of various kinds we have found it advisable to inoculate original material into at least the following three media under vaseline in test tubes, (1) cooked meat medium, (2) dextrose bouillon, and (3) sugar-free bouillon. Sterile tissue may be added to the last two if desired.

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AN EPIDEMIOLOGICAL STUDY OF RHINITIS (CORYZA) IN CALVES WITH SPECIAL REFERENCE TO PNEUMONIA.

BY F. S. JONES, V.M.D., AND RALPH B. LITTLE, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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During the months of November and December, 1921, an outbreak of pneumonia occurred among calves in a dairy herd. This herd has been under observation since 1917 and from previous studies the organisms associated with pneumonia have been established. Theobald Smith[1] in 1917 noted cases of pneumonia among the calves. During 1919 he studied twelve cases of calf pneumonia. In nine instances *Bacillus actinoides* was found. *Bacillus bovisepticus* was also associated with three or four of these. During the latter part of October and throughout November, 1920, there occurred a severe outbreak of pneumonia[2] in adult cows which had been shipped from Michigan. The disease spread to other newly purchased cows and to local and native stock. The outbreak subsided in the dairy herd but cases were observed among the native calves. A few calves born of the Michigan cows had been taken to the calf barns. It was assumed that these calves may have carried the virus.

It has been possible by microscopic and bacteriological examination of the nasal exudate of calves to throw some light on the means of dissemination of the virus and the method by which it may be maintained over considerable periods.

In previous publications Jones[3,4] was able to show that the organisms associated with the outbreak in the cows and infections in calves

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[3] Jones, F. S., *J. Exp. Med.*, 1921, xxxiv, 561.
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occurring throughout the late fall of 1920 and the early months of 1921 differed from those obtained by Theobald Smith during 1917, 1919, and 1920. For convenience they may be referred to as *Bacillus bovisepticus* Groups I and II. Both types are morphologically similar. The groups may be characterized as follows:

Group.	Acid production in.					Hemolysis.	Bile solubility.	Indole.
	Dextrose.	Lactose.	Saccharose.	Maltose.	Mannitol.			
I Associated with outbreak in 1920 and sporadic cases during 1920 and 1921	+	+	+	+	+	+	−	−
II Associated with *actinoides* pneumonia in 1917, 1919, and 1920	+	−	+	−	−	−	+	+

Immunological grouping by means of agglutination affinities followed the cultural grouping. The serum of a rabbit injected with a single Group I strain agglutinated all the Group I cultures but failed to affect those of other groups. A specific agglutinin was also produced for Group II. In no instance was cross-agglutination observed.

It has been stated that during November and December, 1921, there occurred in the calves a small epidemic of pneumonia. Calves which presented no symptoms of pneumonia were frequently found to be suffering from a coryza accompanied by a mucoid or mucopurulent discharge. A number of those showing characteristic symptoms of pneumonia also exhibited a purulent nasal discharge. The discharge from several was examined and organisms possessing all the characters of Type I were obtained. The findings warranted a more complete study. Examinations of the nasal exudate of all the calves in the barn were undertaken.

Method of Examination.

A sterile swab was inserted into both nostrils for a distance of 6 to 8 cm. Films were then prepared on sterile cover-slips. The swab was agitated briskly in 2 cc. of sterile NaCl solution. From this

suspension blood agar plate cultures were made. At first mice were injected beneath the skin with small amounts of the suspension and cultures made from lesions developing about the site of injection. The results were on the whole of less value than those obtained by the plate method, especially since Group I possesses little pathogenicity for any of the laboratory animals. The hemolytic properties of Type I in blood agar plate cultures render its recognition easy. In addition, it is possible to formulate a fair idea of the proportion of *bovisepticus* to other organisms. That the methods employed are not such that it would be possible to detect *Bacillus bovisepticus* in small numbers is admitted, but it was necessary to utilize the material as quickly as possible especially as young calves, probably bringing new infections, were constantly introduced into the barns.

Colonies surrounded by the narrow zone of hemolysis were fished from the plate cultures. If on the microscopic examination the subcultures resembled *Bacillus bovisepticus*, transfers were made to plain agar. After suitable incubation the growth was suspended in 0.85 per cent NaCl and tested against Group I serum. When a culture agglutinated with the serum the fermentation characters were defined.

Material obtained on the swabs must be utilized as quickly as possible. The moist swabs standing at room temperature soon become overgrown with bacteria. Even when swabs are stored in the refrigerator the number of specific organisms diminishes rapidly.

Result of Examinations.

A diagram of the arrangement of the calves in the barn will serve to illustrate the proportion of calves suffering with pneumonia and those which were carrying *Bacillus bovisepticus* in the nasal passages (Table I). The pens are separated from each other by an iron fence with spaces 4 or 5 inches between the uprights.

It will be noted that of the thirty-two calves originally in the barn, two died of pneumonia. In both instances typical organisms of Type I in pure cultures were isolated from the consolidated portions of the lung. In these cases bacteriological examinations of the nasal passages were not made. Of the remaining thirty calves, eight developed clinical manifestations of pneumonia. Of these, four showed a characteristic nasal discharge from which Group I organisms were

TABLE I.

Arrangement of Calves in Pens during the Outbreak.

1. Calves 3 to 4 wks. old.	2. Calves 1 mo. to 5 wks. old.	3. Vacant.	4. Calves 2 to 3 mos. old.	5. Calves 3 to 4 mos. old.	6. Calves 4 to 5 mos. old.	7. Calves 6 mos. old.
1236 Rhinitis.	1231 Pneumonia.		1226	1221	1215 Pneumonia; rhinitis.	1209
1237	1232		1227 Pneumonia.	1222 Rhinitis.	1216	1210
1238 Rhinitis.	1233 Pneumonia; rhinitis.		1228	1223 Pneumonia; rhinitis.	1217 Rhinitis.	1211
	1234 Rhinitis.		1229 Pneumonia; rhinitis.	1224	1218 Pneumonia.	1212
	1235 "		1230 Rhinitis.	1225	1219 Rhinitis.	1213
	675 Pneumonia (died).		676 Pneumonia (died).		1220 Pneumonia.	1214
						673 Rhinitis (introduced Nov. 18, 1921).

cultivated. Eight other calves failed to show symptoms of pneumonia but developed a mucopurulent nasal discharge. From these exudates Type I organisms were cultivated. It will be noted that the oldest calves in Pen 7 all remained free from pneumonia and none developed rhinitis. Calf 673 will be referred to later when certain of the experimental observations are discussed.

The general symptoms of the calves with pneumonia have been noted in a previous communication. There is little disturbance in the general health of calves suffering only from the nasal infection. The temperature may be slightly above the normal. The appetite remains good and there is no apparent digestive disturbance. The calf may occasionally cough or sneeze, forcing out a variable quantity of exudate. The exudate is composed of mucus and masses or strings of polymorphonuclear leucocytes. *Bacillus bovisepticus* is found in varying numbers, often making up from 80 to 90 per cent of the organisms in the plates. At times there was no visible exudate about the nostrils. The introduction of the swab, however, may induce sneezing and lead to the expulsion of exudate. In many instances the nostrils are kept clean by licking so that on casual examination exudate was not observed although the calf suffered with a more or less severe rhinitis.

It is customary on this farm to group calves of the same age in one pen as far as practicable. After they have reached a certain age they are removed to another barn. So a steady stream of calves progresses through each barn. The barn in which the outbreak occurred was quarantined during the epidemic. After the disease had subsided young calves, the majority of which had been injected twice with killed cultures of Group I, were introduced according to routine. During the epidemic board partitions had been erected between certain pens but there remained ample opportunity for contact infections. The next twenty-four calves introduced into this barn were examined at frequent intervals. Among the first lot of vaccinated calves introduced one was suffering from rhinitis. Plate cultures failed to show the hemolytic colonies characteristic of Group I, although a large proportion of the colonies were similar. Suspicion was at once aroused that another group had been introduced. Such proved to be the case. A typical Group II organism was identified.

From this time onward practically all calves placed in this barn developed a rhinitis. In twenty-one instances cultures which fall into Group II have been obtained.

It has been found that the solubility in ox bile of this class of organisms has been of assistance in making identifications. As soon as these organisms were recognized, killed cultures of Group II were included in the vaccine. The nasal infections with Group II continued and were apparently not influenced by the vaccine.

Group I organisms reappeared and were recognized in certain of the newly introduced unvaccinated calves 36 days after the quarantine was lifted. A fatal case of pneumonia developed and two cases of rhinitis occurred. The source of this infection is obscure.

EXPERIMENTAL.

Although the bacteriological and microscopic findings indicated the presence of *Bacillus bovisepticus* within the nasal passages associated with a characteristic purulent exudate, the possibility of other organisms as the primary etiological agent could not be excluded. More complete studies than those made on the spontaneous cases were desirable. With this in view a number of experiments were undertaken.

Experiment 1.—Calf 698. Born Dec. 6, 1921. Dec. 21. The mucosa of both nasal passages was brushed with sterile swabs, films and salt solution suspensions for plate cultures were made from each swab. The films when stained with methylene blue failed to show leucocytes or organisms resembling *B. bovisepticus*. Methods similar to those employed in the herd observations were used in the experimental studies. It was felt that by suspending the material from the swabs in the same amount of salt solution at each examination and using the same amount of the suspension for the preparation of the plates an adequate idea of the increase or decrease of the organisms could be obtained. The cultures used for inoculation were all isolated within a month. They had been passed through three successive passages on horse blood agar.

Dec. 22, 5.15 p.m. The septum of the right nasal passage was brushed with a sterile swab which had been dipped in an 8 hour bouillon culture and then brushed over the surface of a blood agar culture of *B. bovisepticus* (Type I) obtained from the nasal passages of a calf.

Dec. 23. The calf appeared well. There was no visible discharge from the nostrils. Insertion of a sterile swab into the right nostril induced sneezing. A considerable mass of yellowish white, viscid material was ejected. The material

was composed largely of densely packed masses of leucocytes. Characteristic bipolar encapsulated rods were observed in the stained films. Blood agar plate cultures from the suspension revealed a moderate number of Type I colonies in practically pure culture. Material from the left nostril failed to show either leucocytes or *B. bovisepticus*. The highest temperature recorded on this day was 39.1°C.

Dec. 24. There was considerable purulent discharge from the right nostril. Stained films revealed masses of leucocytes and characteristic *bovisepticus* forms in large numbers. The plate cultures revealed numerous characteristic colonies. Films from the left nostril revealed a few leucocytes. The plate cultures did not show colonies of *B. bovisepticus*. The temperature was normal.

Dec. 25. Considerable purulent discharge from both nostrils was noted. The films from the right nostril revealed leucocytes in enormous numbers. The plate cultures showed more colonies than on the preceding day. Preparations from the left nostril contained leucocytes in considerable numbers. In certain instances polymorphonuclear leucocytes which had taken up characteristic rod-like organisms were encountered. Colonies of *B. bovisepticus* did not develop in the plate cultures. The temperature varied between 38.5° and 38.8°C.

Dec. 26. Purulent exudate from both nostrils. *B. bovisepticus* in large numbers in plate cultures from the right nostril.

Dec. 26 and 27. A maximum temperature of 40°C. was recorded.

Dec. 28. The condition was unchanged. About the same number of colonies of *B. bovisepticus* in the plates from the right nostril. After this date the temperature gradually declined until the normal was reached.

Jan. 3, 1922. Right nostril showed purulent exudate. Film showed leucocytes in great numbers and a moderate number of the organisms. Plate cultures showed about the same number of colonies observed Dec. 28. There was no visible exudate from the left nostril. Films revealed very few leucocytes. The plates were negative.

Between Jan. 3 and 10, the amount of exudate from the right nostril gradually diminished. On Jan. 10, material obtained from this nostril contained only an occasional leucocyte. *B. bovisepticus* was not observed in the films nor obtained from plate cultures.

Jan. 11. The calf was reinoculated in the left nostril with the same culture. A purulent discharge was observed on Jan. 12. *B. bovisepticus* was observed in the films and plate cultures but in small numbers. The nostrils were examined daily for the next few days but *B. bovisepticus* could not be obtained from the plate cultures.

During this experiment the general condition of the calf was unaffected. There was, however, a slight temperature disturbance during the height of the disease.

Experiment 2.—The preceding experiment was repeated. Jan. 11, 1922. Calf 697 (aged 43 days) was inoculated in the left nasal passage with a sterile swab dipped in culture. Within 24 hours there was considerable purulent discharge from both nostrils. The organisms were found in the exudate from both nostrils

the 2nd day after inoculation. The greatest numbers were recorded between the 2nd and 5th days. The last record of their presence in plate cultures was on the 12th day. Of interest is the fact that *B. bovisepticus* appeared in the plates in relatively pure cultures. It is to be expected that a few air forms, streptothrix and molds, will be cultivated from the nasal passages of herbivora since they make up a considerable proportion of the organisms found in hay and straw. After the 12th day all examinations were negative. On the whole, the conditions seemed more severe in the case of this calf. The exudate was more copious. The general condition remained unchanged although slight temperature disturbances were recorded.

An opportunity was afforded for observations on spontaneous transmission in the instance of Calf 673 (Table I). The calf was known to be free from Type I infection when placed in a pen adjoining calves showing characteristic rhinitis. Within 3 days there was a slight temperature disturbance. Examination after 5 days showed the characteristic purulent discharge. Plate cultures from the nasal exudate revealed that 70 per cent of the organisms were Type I *bovisepticus*. This calf never manifested symptoms of pneumonia. The organisms subsequently disappeared from the nasal passages.

These experiments clearly establish the findings within the herd that the rhinitis met with during the small outbreak of pneumonia may be attributed to infections with Group I *bovisepticus*.

We have called attention to our failure to find Type I *bovisepticus* in the nasal passages of freshly introduced calves after the outbreak had subsided. The rhinitis continued throughout the months of December and January but in nearly every instance organisms recognized as Group II were obtained. In a number of instances calves recently introduced into these pens would fail to show either leucocytes or *Bacillus bovisepticus* II in the nasal secretion. A week later many of them would have developed a purulent nasal discharge and *Bacillus bovisepticus* II would appear in the plates. Injection with a vaccine containing killed cultures of this organism failed to protect against such infections. To ascertain whether the organisms of Group II were capable of causing these manifestations, two calves were inoculated as in the preceding experiments. Pure cultures recently obtained from the nasal discharges were employed.

Experiment 3.—Jan. 26, 1922. The mucosa of the right nostril of Calf 715B (21 days old) was brushed with a swab immersed in an 18 hour bouillon culture and

then smeared over the surface of an 18 hour blood agar slant of a typical Group II strain.

Jan. 27. There was no visible exudate but films from the swab showed leucocytes in masses and a few *bovisepticus*-like forms. The plate cultures failed to show suggestive colonies. The films from the left nostril revealed only epithelial cells and a few cocci.

Jan. 28. There was no visible exudate. The swab from the right nostril showed purulent exudate. Leucocytes in masses and a moderate number of characteristic rods were present in the films. The plates revealed a considerable number of colonies, at least 90 per cent of which resembled *bovisepticus*. Material from the left nostril did not contain leucocytes or *B. bovisepticus*.

Jan. 30. The right nostril contained a small quantity of thick, yellow, purulent exudate containing leucocytes in masses and large numbers of characteristic rods. The plate cultures were largely made up of *B. bovisepticus* II. The secretion from the left nostril failed to contain leucocytes or characteristic organisms.

Jan. 31. There was purulent exudate on the swab from the right nostril. The organisms were more numerous in the plate cultures.

Feb. 2. There were leucocytes in the material obtained on the swab from the right nasal passage. The plate cultures showed only about half as many colonies as on Jan. 31.

Feb. 6. Relatively few leucocytes in the secretion from the right nasal passage. *B. bovisepticus* still made up 80 per cent of the colonies. The swab from the left passage failed to show leucocytes or characteristic colonies.

Feb. 10. *B. bovisepticus* still persisted in the right nostril. They were still present on Feb. 15.

Experiment 4.—Calf 757, 16 days old when inoculated in the left nasal passage with Type II culture obtained from the nasal exudate of a calf. In general the course of the infection paralleled that of Calf 715B. The exudate was purulent during the first 4 days. The organisms were most numerous 3 days after inoculation. After 5 days the exudate contained only a few leucocytes but still contained the organism. From the right nasal passage it was not possible to obtain *B. bovisepticus* in the plates.

Neither of these calves showed a systemic reaction. The temperature remained well within the normal limits.

From the experiments it appeared that Group I organisms when introduced into the nasal passages increase in number for 4 or 5 days. After this maximum is reached there is a sharp decline and in most instances the organism can no longer be cultivated after 10 to 18 days. With Group II the organisms persisted after the disease had subsided.

If it were possible to show that organisms of both groups were not entirely eliminated from the nasal passages in certain individuals

after considerable periods, it would be possible to explain the sudden appearance and disappearance of pneumonias in the herd. With this in view all calves which had suffered from rhinitis were reexamined during the latter part of February and early part of March, 1922. In two cases (Calves 1234 and 1235, Table I) Type I organisms were still present in the nasal passages 113 and 121 days after their first discovery. In another instance a calf infected during January, 1922, was known to have carried the organisms for a period of 36 days.

About the same proportion of calves continued to carry Group II organisms. Of sixteeen calves reexamined, four continued to harbor Group II organisms within the nasal passages after periods of 50, 66, 66, and 73 days. It seems reasonable to conclude that certain calves may become carriers of both groups for indefinite periods. It must be borne in mind that the methods employed are such that carriers of small numbers of organisms may be overlooked.

DISCUSSION.

The observations are of considerable interest in certain respects. It is possible to divide the calves in the outbreak associated with *bovisepticus* Group I into four lots. Those that were most susceptible to the infection died of diffuse pneumonia. A larger number, more resistant perhaps, developed severe clinical manifestations and apparently recovered. Still others failed to develop any symptoms of grave respiratory disturbance but suffered from a coryza. These animals were exposed to the same general environment as the others but for some reason were more resistant. Scattered throughout the pens were other calves that failed to present any manifestations of infection. In addition to these there remain the oldest calves in Pen 7. Group I organisms were not found in the nasal passages nor was pneumonia observed among them. These calves were born in May and June, 1921. They may have been exposed to cases in these months and developed a certain degree of immunity. It is known that sporadic cases occurred in this barn during the spring of 1921.

Of greater interest is the ability of both types of organisms to exist in specific instances on the nasal mucous membrane. From the epidemiological point of view this is of considerable significance. Group I is capable of producing a severe pneumonia or a mild infection

of the nasal passages. Two calves which suffered only from the rhinitis have continued to carry these organisms in the nasal passages for several months. It is possible to explain the sudden outbreaks in calves in the winter months to exposure and infection from the mild cases of rhinitis or to carriers. The same explanation may be applied to some of the outbreaks among cattle which have been shipped. Often cows and steers for shipment are purchased from several sources. After they have been assembled they are loaded and shipped. The inclusion of a carrier or a mild case of rhinitis would afford ample opportunity for infection. This is especially true when the conditions of shipment are considered. There is ample opportunity for considerable lowering of resistance, especially during the colder months of the year.

Newsom[5] in discussing the various forms of hemorrhagic septicemia in sheep describes an acute type (septicemic) and the more chronic types, such as the pulmonary, enteric, and cerebral forms. For the sake of completeness he calls attention to a chronic catarrhal disease of the nasal passages of sheep, although he states that he has no definite knowledge that it is a form of hemorrhagic septicemia.

There are few data relative to the pathogenicity of organisms belonging to Group II. In the observations on this particular herd they have not been regarded as the prime factor in pneumonias of either cows or calves. Further observations to establish their relation to pneumonias of calves are necessary. They have been found by Theobald Smith in calves infected with *Bacillus actinoides*. It has been possible to show that Group II is associated with a mild rhinitis which is communicable from infected to healthy calves.

Some of the characters possessed by both types render their recognition easy. The narrow hemolytic zones about the deep colonies of Type I in horse blood agar plates are an important character. After the study of many strains it was found that cultures of the proper morphology which were hemolytic and agglutinated with the Group I serum possessed the fermentation characters of the cultural group. Bile solubility affords a ready method of distinguishing the members of Group II. From present indications it seems safe to consider

[5] Newsom, I. E., *23rd Ann. Rep. U. S. Livestock San. Assn.*, 1919, 203.

that those organisms which are bile-soluble and agglutinate with Group II serum will fall into a specific cultural group.

Early in the course of the study it was hoped that it would be possible to type the organisms obtained by means of swabs from outbreaks occurring some distance from the laboratory. It was found that material obtained on the swabs rarely showed viable *bovisepticus* types after 18 to 24 hours in the refrigerator. Material standing at room temperature was soon overgrown with other organisms and became valueless for diagnostic purposes.

SUMMARY.

During the month of November there occurred an outbreak of pneumonia among the calves in a large dairy. Thirty-two calves in one barn were exposed to the disease. Ten clinical cases developed. Two died of diffuse pneumonia. From these *bovisepticus* Group I organisms were obtained at autopsy. Four affected with pneumonia and eight other calves which failed to show symptoms of pneumonia developed a purulent rhinitis. From the nasal exudate of these cases Group I organisms were cultivated. The characteristic rhinitis was reproduced experimentally by brushing the nasal mucosa with a swab dipped in culture. Certain of the calves which suffered from the spontaneous rhinitis continued to carry the organisms in the nasal passages for periods as long as 121 days.

After the first outbreak had subsided practically all calves introduced into this barn developed a milder type of rhinitis associated with organisms of Group II *bovisepticus*. 25 per cent of such calves continued to carry the organism on the nasal mucosa for periods of 50 to 73 days. It was possible to induce nasal infection in calves with pure cultures of this organism.

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THE RELATION BETWEEN THE ACCUMULATION OF GLOBULINS AND THE APPEARANCE OF AGGLUTININS IN THE BLOOD OF NEW-BORN CALVES.

BY MARION L. ORCUTT AND PAUL E. HOWE, PH.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N.J.)

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Recent work has demonstrated with regard to the agglutinins of *Bacillus abortus* that the blood of the normal new-born calf before it has received colostrum does not contain agglutinins. If the calf receives colostrum from a cow with a high titer for *Bacillus abortus*, agglutinins will appear in the blood in a relatively short time (1). Furthermore, the blood of a new-born calf before it has suckled does not contain appreciable quantities of protein precipitated by concentrations of sodium sulfate which are less than 17.4 per cent whereas after it has ingested colostrum these proteins will be present in relatively large amounts (2). The ingestion of milk does not result in the increased appearance of the two globulin fractions of the blood. The data presented in this paper bear upon the relation which exists between the appearance of the agglutinins and the accumulation of globulins in the blood of new-born calves and upon the protein fractions in the colostrum and blood with which the agglutinins are associated.

I.

The Simultaneous Appearance of Agglutinins and Certain Globulins in the Blood of New-Born Calves.

Presentation of Data.—The method of collecting the samples of blood, and the determination of the agglutinin titer of blood and colostrum have already been presented (1, 3). The procedure for the determination of the proteins of blood and colostrum has also been

given (2, 4, 5). The accompanying tables contain representative data.

The data contained in Table I illustrate the rapidity with which the agglutinins and globulins are absorbed by the new-born calf. In the first case (calf of Cow 664), an agglutinin titer of 1:80 is obtained

TABLE I.

*Data Showing the Changes in the Agglutinin Titer and in the Proteins of the Blood of Calves Following the Ingestion of Colostrum.**

Age of calf.	Total nitrogen.	Euglobulin.	Pseudoglobulin I.	Pseudoglobulin II.	Total globulin.	Albumin.	Non-protein nitrogen.	Agglutination.
Calf of Cow 664.								
20 min.	0.776	0.018	0.072	0.184	0.273	0.430	0.073	—
Calf allowed to suckle dam.								
4 hrs.	0.896	0.098	0.218	0.115	0.431	0.391	0.073	1:80
6 " 10 min.	1.066	0.162	0.303	0.183	0.648	0.354	0.064	1:160
14 " 30 "	1.399	0.512	0.401	0.175	1.088	0.247	0.064	1:320
11 days.	1.352	0.333	0.469	0.149	0.951	0.367	0.034	
Calf of Cow 411.								
Dam's blood.	1.135	0.090	0.286	0.119	0.495	0.606	0.034	
35 min.	0.759		0.068	0.154	0.222	0.460		—
Calf allowed to suckle dam.†								
1 hr., 50 min.	0.700		0.073	0.149	0.222	0.422	0.077	1:40
3 hrs., 50 "	0.802	0.038	0.094	0.171	0.303	0.422		1:640
18 "	0.925	0.085	0.290	0.153	0.443	0.354	0.043	1:2,560

* Results in this and the accompanying tables are expressed as grams of nitrogen in 100 cc. of blood, and as the highest dilution at which agglutination was positive.

† The composition of the first colostrum was as follows, expressed as grams of nitrogen per 100 cc. of centrifuged colostrum: total nitrogen 2.747, euglobulin N 0.899, casein N 1.097, pseudoglobulin I N 0.03, pseudoglobulin II N 0.154, albumin N 0.224, non-protein N 0.074.

within less than 3 hours. In the same time the amount of pseudoglobulin I has increased from 0.072 gm. of nitrogen per 100 cc. of blood to 0.218 gm. of nitrogen and the euglobulin has increased in a like proportion; about 2 hours later analyses showed further increases in both the agglutinin titer and the globulin content of the serum.

In the second case (calf of Cow 411), the increase in the globulin content is not as rapid but the agglutinin titer is much higher. With both animals an absorption of agglutinins is accompanied by an increased amount of globulin in the blood.

The effect of withholding colostrum from calves for a short period of time is shown in the first portions of the data contained in Table II. From an inspection of the data it is evident that when colostrum is withheld from a calf and ordinary milk is given, neither the agglutinins nor the globulins appear in the blood to an appreciable extent for at least 21 hours. In this time a calf which has received colostrum soon after birth will have accumulated a considerable quantity of globulin in its blood and also agglutinins, if they are present.

When colostrum is fed at the end of 17 or 21 hours, in three cases[1] agglutinins appear in the blood and there is a very definite increase in the concentration of euglobulin and pseudoglobulin I (calf of Cow 3401, Table II) and of euglobulin and pseudoglobulin in the case of the calf of Cow 627.[2]

There is a difference between the calf of Cow 3401 and the calf of Cow 627 in that in the first case the calf received milk which had been refrigerated for approximately 17 hours and then warmed, and later another milking of colostrum was fed which had been held only 8 hours before feeding. The calf of Cow 627 suckled another cow (No. 626) immediately after bleeding at 21½ hours. The agglutinin titer of the blood in each of these cases reflects the titer of the colostrum which the calf ingested.

[1] A third case is not reproduced here but is to be found in a previous publication (2), Calf B. This calf received 36 ounces of colostrum, which had been refrigerated and warmed before it was fed, approximately 21 hours after birth, and later 92 ounces of colostrum at approximately 28½ hours after birth. The agglutinin titer of the blood serum was negative at birth and first gave a reaction at 28½ hours, 1:160; at 45 hours the titer was 1:160, and at 71 hours it was 1:320.

[2] At the time the blood of this calf was analyzed the presence of two pseudoglobulins had not been established for the analtyical procedures used, and analyses were not made at 17.4 per cent of sodium sulfate. The data presented represent pseudoglobulins I and II. Analyses made later on these samples showed the presence of pseudoglobulin I following the ingestion of colostrum and the essential absence of this protein before receiving colostrum. The data are not given since they are definitely open to question.

TABLE II.

Data Showing the Changes in the Agglutinin Titer and in the Proteins of the Blood of Calves Following the Ingestion of Colostrum. Effect of Withholding Colostrum for a Number of Hours.

Age of calf.	Total nitrogen.	Euglobulin.	Pseudoglobulin I.	Pseudoglobulin II.	Total globulin.	Albumin.	Non-protein nitrogen.	Agglutination.
Calf born of a low titer cow (No. 3401), restrained from taking colostrum until after two blood examinations, then fed colostrum which had been refrigerated and warmed. Titer of colostrum 1:160.								
1 hr., 20 min.	0.805	0.000	0.001	0.102	0.289	0.463	0.053	—
17 hrs., 20 "	0.693	0.076	0.000	0.153	0.229	0.416	0.048	—
Fed 96 ounces of colostrum which had been refrigerated and warmed.								
21 hrs., 50 min.	0.812	0.068	0.131	0.110	0.309	0.459	0.044	1:20
Fed colostrum at 1.30 p.m. taken from dam at 5.30 a.m.								
26 hrs.	1.046	0.131	0.228	0.149	0.508	0.481	0.057	1:40
44 "	0.954	0.144	0.298	0.048	0.490	0.394	0.070	1:40
68 "	0.854	0.097	0.245	0.039	0.381	0.407	0.066	1:20
Calf of Cow 627. Restrained from taking colostrum until after three examinations of blood, during which time it was fed milk free from agglutinins, then allowed to suckle a high titer cow (No. 626) which had given birth to a calf the same day. Titer of colostrum 1:2,560.								
1 hr., 50 min.	0.652	0.053		0.120*	0.173	0.416	0.052	—
7 hrs., 10 "	0.598	0.047		0.175	0.222	0.328	0.048	—
21 " 30 "	0.638	0.034		0.173	0.207	0.378	0.053	—
Calf placed with Cow 626 immediately after bleeding.								
32 hrs.	0.648	0.136		0.170	0.306	0.302	0.035	1:640
46 " 50 min.	0.772	0.142		0.248	0.380	0.339	0.053	1:640
71 " 20 "	0.839	0.191		0.186	0.377	0.409	0.053	1:640
79 " 50 "	0.778	0.130		0.178	0.308	0.431	0.039	1:1,280
93 " 20 "	0.774	0.135		0.164	0.299	0.422	0.053	
Calf born of a high titer cow (No. 634), restrained from taking colostrum until after three blood examinations, during which time it received milk free from agglutinins, then fed the dam's colostrum which had been refrigerated and warmed. Titer of colostrum before refrigeration 1:640.								
1 hr.	0.740	0.035		0.252*	0.287	0.400	0.053	—
6 hrs.	0.774	0.021		0.271	0.292	0.438	0.044	—
22 " 30 min.	0.660	0.039		0.166	0.205	0.407	0.048	—
Calf received 33 ounces of colostrum immediately after bleeding.								
29 hrs.	0.791	0.126		0.197	0.233	0.420	0.048	—
45 "	0.772	0.096		0.140	0.236	0.483	0.053	—
53 "	0.843	0.077		0.200	0.277	0.523	0.053	—
3 days.	0.832	0.094		0.212	0.268	0.489	0.044	—
4 "	0.717	0.065		0.175	0.240	0.429	0.048	—
5 "	0.740	0.084		0.175	0.259	0.438	0.043	—
7 "	0.730	0.095		0.184	0.279	0.407	0.044	—

*The remaining data in this column refer to pseudoglobulin I plus pseudoglobulin II.

The third case in this series (calf of Cow 634) received colostrum which had been refrigerated for approximately 22 hours, and showed but a slight increase in the globulin content of the blood and no agglutinins. The remarks just made with regard to the analysis of the blood of the calf of Cow 627[2] apply to this case. The globulin content of the colostrum was not determined. It is impossible to say, therefore, whether the failure to obtain a marked rise in the globulin content of the blood serum was due to the low globulin content of the colostrum, to the effect of refrigeration, or to the age of the calf. The latter condition would seem to be ruled out by the results of the other two experiments and on Calf B previously reported (2). Subsequent data indicate that the composition of the colostrum does have a relation to the kind and quantity of globulin which appears in the blood.

DISCUSSION.

A definite relation between the absorption of globulins and the absorption of agglutinins by new-born calves is brought out in the data presented. The absorption of euglobulin and pseudoglobulin I[3] is a process independent of the presence or absence of agglutinins for *Bacillus abortus*. Whether or not agglutinins can be obtained, and absorbed independent of the globulins we do not know, but it appears doubtful from the work of investigators who have studied the chemistry of immunity. We have fractioned samples of milk with a high agglutinin titer which did not contain a marked increase in globulin content, but the agglutinins were removed by concentrations of salt which remove the globulins.

From the data presented it is evident that the agglutinins which the new-born animal acquires by the ingestion of colostrum are associated with the direct absorption of the globulins of colostrum.

There are numerous references in the literature to the absorption of unchanged foreign protein based upon biological tests. With regard to the absorption of colostrum we have the work of Langer, Bauer, and Bauereisen. Langer (6)

[3] By euglobulin is meant protein precipitated from serum or colostrum at 14.0 to 14.2 per cent of sodium sulfate, *i.e.* 14.2 gm. of sodium sulfate contained in 100 cc. of solution at 37°C., and by pseudoglobulin I is meant protein precipitated between 14.2 and 17.4 to 18.4 per cent of sodium sulfate and exclusive of casein in the case of colostrum.

demonstrated, by means of the precipitin reaction, the absence of substances in the blood of the new-born calf which would react with colostrum antiserum, whereas colostrum antiserum and cow blood did react. After suckling, and as early as 6 hours after suckling, substances appeared in the blood of the calf which reacted with colostrum antiserum. The maximum content of reacting substance was reached on the 2nd day, after which the values were relatively constant. A new-born calf allowed to suckle a cow which had given birth 3 weeks previously did not have in its blood, after 48 hours, substances which would react with colostrum antiserum. Langer concluded that the blood of the new-born received from colostrum certain additions by direct absorption which made the blood similar in composition to that of its mother.

Bauer (7) presented evidence for new-born infants, based upon the complement fixation test, similar to that of Langer's. He concludes, however, that since all children contain the acquired substance within the first 2 months no matter what the diet may be, that the acquired protein is formed and increases during early life. On the other hand, Bauer used antiserum to the soluble proteins of cow's milk and obtained reactions with the blood of the new-born calf which had not suckled. From this observation he concluded that a calf at birth is more advanced in its development than the infant.

Bauereisen (8) studied, with the precipitin reaction, the problem of the relation of blood of the new-born to the blood of the mother, to colostrum, and to milk. He conducted his experiments with reacting substances of equal protein content, since the blood of the new-born is low in protein, and likewise milk, as compared with colostrum. Under such conditions Bauereisen found the blood from the umbilical cord of calves to give identical reactions with the antisera of colostrum, milk protein, and casein. Antisera of blood obtained from the umbilical cord did not give a high titer. However, colostrum antiserum precipitated the blood from the umbilical cord of infants. He concludes, therefore, that the substances present in the blood of the mother are also present in the blood of the unsuckled new-born infant. The difficulties of the previous investigators had been in the difference in the protein content of the two bloods.

It is to be remembered that in the work just reviewed the investigators were working with mixtures of proteins, with the exception of casein, and some of the data suggest that even the casein was impure. Bauereisen was probably correct in his deductions from his experimental data, but from the results presented here and that of Howe (2) it is evident that Langer was correct in assuming that the new-born animal receives from colostrum certain additions to its blood. These additions are, as we have shown, globulin fractions, the fractions which carry agglutinins. There are other, quantitative, differences between the serum of the new-born and that of the adult, some of which we hope to present in the near future.

II.

The Protein Fractions of Colostrum and of Serum with Which the Agglutinins for Bacillus abortus Are Associated.

Presentation of Data.—Samples of colostrum and of serum with high agglutinin titers for *Bacillus abortus* were fractioned with sodium sulfate at 37°C. to determine the protein fraction or fractions which would contain the agglutinins. Proteins when precipitated are readily soluble in water and the adherent salt. The general procedure for the separation of protein fractions was to precipitate small quantities, 0.5 to 1.0 cc. of colostrum or serum, with various concentrations of sodium sulfate, wash the precipitate with the concentration of sodium sulfate used in precipitation, and then to dissolve the precipitated protein in a known quantity of distilled water. The final concentration of the protein was then known within approximate limits; the assumption was made that the quantity of water retained in the filter after dissolving the precipitate was the same as that which was present after filtration and washing. The protein solutions contained sodium sulfate in variable amounts which depended upon the concentration of sodium sulfate used. That this salt did not in itself cause agglutination nor inhibit agglutination at the high dilutions was demonstrated by control experiments in which the concentrations of sodium sulfate employed for precipitation were used and by tests upon filtrates from precipitations which completely removed the agglutinins. In some cases the proteins were reprecipitated and again tested for agglutinins. The procedure followed in testing for agglutinins has been described elsewhere (3). Representative data on serum and colostrum are contained in Tables III to IX.

The removal of agglutinins from serum by sodium sulfate precipitation is shown in Tables III and IV. The data in Table III relate to the agglutinin value of the protein precipitated from serum at various concentrations of sodium sulfate, and also of the original filtrates from such precipitations. One set of data is the complement of the other. It is evident that a considerable proportion of the agglutinins is removed by 14.5 per cent of sodium sulfate and that complete removal is accomplished at 16.4 per cent of sodium sulfate. Similar results are shown in Table IV. In this case, however, instead of

TABLE III.

The Agglutinin Titer of Protein Fractions from Serum, Separated with Sodium Sulfate at Definite Concentrations, and of the Filtrates from These Separations.

Fractions.	Dilutions.						Control.
	1:20	1:40	1:80	1:160	1:320	1:640	
Serum.	C.	C.	++ ++	++	+	±	–
Na_2SO_4, per cent							
13.5		++	+	±	–	–	–
14.2		++	++ +	+	–	–	–
14.5		++ ++	++ ++	++	+	–	–
15.5		++	++ +	++ +	+	–	–
16.4		++ +	++ +	++ +	++	–	–
17.4		++	++ +	++	++	–	–
21.5		++	++ ++	++	+	–	–
Filtrates.							
13.5 F		C.	C.	++ +	+	–	–
14.2 F		"	++ +	+	–	–	–
14.5 F		++ ++	++	–	–	–	–
15.5 F		++ ++	+	–	–	–	–
16.4 F		+	–	–	–	–	
17.4 F		–	–	–	–	–	–
21.5 F		–	–	–	–	–	–

examining the filtrate from the original precipitation directly, the agglutinin titer of the protein precipitated at 18 per cent of sodium sulfate was employed; at this concentration all agglutinins have been

TABLE IV.

*The Agglutinin Titer of Protein Fractions from Serum Which Were Precipitated with Sodium Sulfate and of the Protein in the Filtrates Precipitated by Increasing the Concentration of Sodium Sulfate to 18 Per Cent.**

Fractions.	Dilutions.								Control.
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	
Serum.	++ ++	++ ++	++ +	++	++ +	++ +	++ +	++	−
Na_2SO_4, *per cent*									
13.5		++	++	+	±	−	−	−	−
14.5		++ +	++ +	++	+	±	−	−	−
15.5		++	++ +	++	++	±	−	−	−
17.4		++ +	++ +	++	++	−	−	−	−
Filtrate protein.									
13.5/18		++ +	++ ++	++	+	−	−	−	−
14.5/18		++ +	+	−	−	−	−	−	−
15.5/18		−	−	−	−	−	−	−	−

* The solutions of the precipitates produced by 14.5, 15.5, and 17.4 per cent of sodium sulfate contained a dark precipitate the following day which was removed by centrifugation.

removed in every case studied; in fact, 16.4 per cent of sodium sulfate is usually sufficient.

Data relating to the removal of agglutinins from colostrum are contained in Tables V to IX. The data in Table V illustrate the

TABLE V.

The Agglutinin Titer of Protein Fractions from Colostrum, Separated with Sodium Sulfate, and of the Filtrates from These Fractions.

Fractions.	Dilutions.										Control.
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	
Colostrum.	++	++ +	++ +	++ +	++ +	++ ++	++ ++	++ ++	++ +	+	−
Na_2SO_4, *per cent*											
7.5	++	+	−	−	−	−	−	−	−	−	−
9.5	++ ++	++ +	+	−	−	−	−	−	−	−	−
10.5	++ ++	++ +	+	−	−	−	−	−	−	−	−
12.5	++ ++	++ ++	++ ++	++ ++	++ ++	++ +	+	−	−	−	−
13.5	++ ++	++ ++	++ ++	++ ++	++ ++	++ +	++ +	+	−		−
14.2	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	++ +	++	±	−	−
14.5	++ +	++ +	++ +	++ +	++ ++	++ ++	++	+	−	−	−
15.5	++ +	++ +	++ +	++ ++	++ ++	++ ++	++ ++	++ +	+	−	−
16.4	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	++ +	++	+	−
17.4	++ +	++ +	++ +	++ +	++ ++	++ ++	++ ++	++ +	++	−	−
Filtrates.											
7.5 F	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	++ +	+	−	−	−
11.5 F	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	−	−	−	−	−

TABLE V—*Concluded.*

Fractions.	Dilutions.										Control.
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	
	Filtrates.										
Na_2SO_4, *per cent*											
12.5 F	++ ++	++ ++	++ ++	++ ++	++ ++	+	−	−	−	−	−
13.5 F	++ ++	++ ++	++ ++	++ ++	++ ++	++	+	−	−	−	−
14.2 F	++ ++	++ ++	++ ++	++ ++	+	−	−	−	−	−	−
17.4 F	+	−	−	−	−	−	−	−	−	−	−

extent to which the agglutinins are removed by each concentration of sodium sulfate. The agglutinin titers of the precipitates increase up to approximately 16.4 per cent of sodium sulfate. A considerable proportion of the agglutinins is precipitated with the proteins separating at 12.5 per cent of sodium sulfate. The precipitates in these cases were obtained from 1 cc. of colostrum and were washed four times with sodium sulfate solutions of the concentrations used in precipitating them. The titrations of the filtrates from the original precipitations show that the agglutinins have been completely removed at 17.4 per cent of sodium sulfate, a concentration which precipitates all of the protein which we have assumed to be pseudoglobulin I. The demonstration of the complete removal of agglutinins by examination of the protein contained in the filtrates, such as was done for serum (Table IV), is contained in Table VII.

There is a possibility in the procedures followed in separating the protein fractions that the precipitates are contaminated by solution retained on the filter or adsorbed on the precipitate. The experiment detailed in Table VI was conducted in an attempt to obviate this possibility. After precipitating the proteins and washing the precipitates, they were dissolved in water and tested for agglutinins. An aliquot portion of the remaining solution was taken, the protein reprecipitated, and, after again washing thoroughly, the precipitate

TABLE VI.

*The Agglutinin Titer of (a) Protein Fractions Precipitated from Colostrum by Sodium Sulfate and (b) after Reprecipitation of the Same Fractions.**

Fractions.	Nitrogen per 100 cc.	Dilutions.									Control.
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	
	gm.										
Rennet whey (1).	3.439†	++	++	++	++	++	++	++			
			+	+	+	++	++	++			
" " (2).		++	++	++	++	++	++	++	++	++	−
		+	+	++	+	+	+	++	++		
Na_2SO_4, per cent											
13.8 (*a*)	0.742	++	C.	++	++	++	++				−
		++		++	++	+					
13.8 (*b*)		++	++	++	++	++	++	+	−	−	−
		++	++	++	++	+					
14.0 (*a*)	0.998	++	++	C.	++	++	++	++			−
		+	++		++	++	+				
14.0 (*b*)		++	++	++	++	++	++	++	−	−	−
		++	++	++	++	+	+				
14.2 (*a*)	1.006	++	++	++	++	++	++	++			−
					++	+	+	++			
14.2 (*b*)		++	++	++	++	++	++	++	++	+	−
		++	++	++	++	++	++	+			
18.4 (*a*)	2.380	++	++	++	++	++	++	++			−
		+	++	++	++	+	+				
18.4/14.2 (*b*)		++	++	++	++	++	++	++	+	−	−
		++	++	++	++	+	+				
Filtrate from 18.4/14.2		C.	++	+	−	−	−	−	−	−	−

* The first agglutination series and rennet whey (1) extended only to a dilution of 1:1,280. In the case of 18.4/14.2 (*b*) the dissolved protein precipitated at 18.4 per cent of sodium sulfate was reprecipitated at 14.2 per cent of sodium sulfate.

† Colostrum.

was dissolved and tested. It is evident that the agglutinins remained with the protein material.

TABLE VII.

Data Showing (1) the Effect of Clearing Colostrum with Sodium Oxalate, (2) Precipitation with Carbon Dioxide, and (3) the Agglutinins Remaining in Solution after Precipitation of Proteins at Given Percentages Which Are Afterward Precipitable by 18 Per Cent of Sodium Sulfate.

Fractions.	Nitrogen per 100 cc.	Dilutions.								Control.
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	
	gm.									
Colostrum, whey.	2.640*	++ +	++ ++	++ ++	++ ++	++ +	++ +	++	+	−
Colostrum, oxalated.		++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	++ +	++	−
CO_2, oxalated.	1.365	++	++ +	++ +	++ +	++ +	++	+	±	−
CO_2	1.365	++ +	++	++	++ +	++ +	++	+	+	−
CO_2, filtrate.	1.275		+	+	++	++ ++	++	+	±	−
Na_2SO_4, per cent										
13.5/18		++	++ +	++ ++	++ ++	+	−	−	−	−
14.5/18		++ ++	++ ++	++	+	−	−	−	−	−
15.5/18		−	−	−	−	−	−	−	−	−
16.4/18		−	−	−	−	−	−	−	−	−

* Colostrum.

In the course of experiments relating to the determination of the proteins of colostrum, attempts were made to utilize carbon dioxide as an agent for the separation of the proteins. It was found that variable results were obtained, in some cases casein alone was precipitated and in others euglobulin and casein. In Table VII are data

relating to the agglutinin titer of the proteins separated by carbon dioxide, from which it appears that a large proportion of the agglutinins was removed by carbon dioxide. In this case the carbon dioxide removed both casein and euglobulin, a fact which is evident from other

TABLE VIII.

The Agglutinin Titer of Protein Fractions Removed Successively from Solution by Sodium Sulfate or by Acetic Acid.

Fractions.	Nitrogen per 100 cc.	Dilutions.							Control.
		1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	
	gm.								
Colostrum, oxalated.	3.872	++ ++	++ ++	++ ++	++ ++	++ ++	++ +	+	−
Procedure *a*.									
Casein, direct precipitation.	0.648	−	−	−	−	−	−	−	−
Na_2SO_4 14.2 per cent after casein.	0.804	++ +	++ +	++ ++	+	−	−	−	−
Na_2SO_4 17.4 per cent after 14.2 per cent.	1.201	++	++ +	++ ++	++ ++	++ +	+	−	−
Procedure *b*.									
Na_2SO_4 14.2 per cent.	0.993	++	++ +	++ +	++ ++	++ +	++	−	−
Casein after 14.2 per cent.	0.755	−	−	−	−	−	−	−	−
Na_2SO_4 17.4 per cent after 14.2 per cent less casein.	1.126	++ ++	++ ++	++ ++	++ +	+	−	−	−

analytical results obtained at the same time; hence the precipitation of agglutinins is to be compared with those obtained at 14.2 per cent of sodium sulfate. The agglutinin titer of protein not precipitated at 14.5 per cent of sodium sulfate (14.5/18) in the other experiments is to be compared with the data relating to the carbon dioxide precipitation.

Occasionally a sample of colostrum is not coagulated readily by

rennin, which interferes with the titration of the agglutinins. Sodium oxalate will readily remove the opacity of a solution of milk or colostrum, particularly if it be diluted with one or two volumes of a salt solution or water (5). Such additions of sodium oxalate have not interfered with the determination of agglutinins in a number of cases in our experiments. An example is contained in Table VII. On the other hand, the introduction of such a salt into the agglutinating system is not desirable. We have, therefore, used the whey unless it was impossible to do so.

The experiments outlined with regard to colostrum have involved the determination of the agglutinin titer of single proteins or mixtures of proteins precipitated in each case from a definite quantity of the original colostrum. In Table VIII are data which bear upon the removal of agglutinins by the successive precipitation of proteins or groups of proteins from one sample of colostrum. Two portions of the colostrum were used. From the dilute solution of one (*a*) the casein was removed by the addition of acetic acid and then, after careful neutralization, the globulins were precipitated with sodium sulfate. In the second portion (*b*) sufficient sodium sulfate was added to give the solution a concentration of 14.2 per cent, and after filtration the casein was precipitated with acetic acid, the filtrate neutralized, and sodium sulfate added to make a concentration of 17.4 per cent to separate the pseudoglobulin I fraction.

The data indicate that casein does not carry the agglutinins; the precipitated casein was dissolved by the careful addition of 0.1 N NaOH. The discrepancy between the agglutinin titers obtained at 14.2 per cent of sodium sulfate after casein has been removed and before the removal of casein is due, we believe, to the change in the precipitability of the globulins as the result of acidification and neutralization following the direct precipitation of casein from the original solutions of colostrum. That different quantities of protein are precipitated is evident from the different amounts of protein nitrogen present in these cases. This difficulty has been experienced in other experiments relating to agglutinins and in attempts to precipitate the proteins of colostrum (5). The variable results found at 14.2 per cent of sodium sulfate are reflected in the agglutinin titers of the subsequent fractions. The most important result is, however,

the failure to obtain agglutinins with the casein fractions. That the absence of agglutinins is not the result of destruction resulting from acidification and neutralization is evident from the demonstration of agglutinins in the subsequent fractions precipitated by sodium sulfate after neutralization.

TABLE IX.

The Agglutinin Titer of Protein Fractions of Milk.

Fractions.	Nitrogen per 100 cc.	Dilutions.							Control.
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	
	gm.								
Milk, whey.	0.478	C.	++ ++	++ ++	++ +	++	+	−	−
Na_2SO_4, per cent									
13.5	0.022	++ ++	++ +	++	+	−	−	−	−
14.0	0.017	++ ++	++ ++	++	+	−	−	−	−
14.2	0.034	++ ++	++ +	++	±	−	−	−	−
17.4	0.277	++ ++	++ ++	++ +	++ +	++	±	−	−

Certain samples of milk having a relatively high agglutinin titer were available. Fractionation of such samples (Table IX) gave results similar to those found for colostrum. On the other hand, if we exclude the casein the agglutinins are associated with a relatively small amount of protein.

DISCUSSION.

The data presented with regard to the precipitation of agglutinins for *Bacillus abortus* in blood and colostrum indicate definitely that the protein, or protein mixtures, precipitated up to and including 16.4 per cent of sodium sulfate carries with it the agglutinins. It is this fraction, which is absent from the blood of the new-born calf, which is abundant in most samples of colostrum and which is absorbed directly

by the new-born animal. The association of agglutinins with a particular protein fraction is not clear-cut. It appears that a large proportion of the agglutinins is associated with the fraction which is precipitated by 14.2 per cent of sodium sulfate, euglobulin. On the other hand, agglutinins were found in later fractions, 14.2 to 16.4 per cent of sodium sulfate. If we assume that the agglutinins are associated only with the euglobulin fraction, precipitated by 14.2 per cent of sodium sulfate or by carbon dioxide, then it is necessary to conclude that the agglutinins appearing in the later fractions are there because of a failure to precipitate all of the euglobulins in the process of fractionation. The method of separation does not preclude such a possibility. Certain results obtained in testing samples of colostrum for agglutinins, in our search for a high titer colostrum, leave the situation at present just where a literal interpretation of the data given would place it; *i.e.*, that the agglutinins are associated with the protein fractions which are precipitated up to and including 16.4 per cent of sodium sulfate. The observations referred to related to samples of colostrum having a low titer with a euglobulin content which was high or relatively high. In these cases the agglutinins did not appear, or but a trace was present, in the fraction precipitated at 14.0 to 14.2 per cent of sodium sulfate, but did appear in fractions precipitated at 18.4 per cent of sodium sulfate; in these preliminary tests only two concentrations of sodium sulfate were used. It is evident, however, that while there may be difficulties of separation they concern only the euglobulin fraction and the pseudoglobulin I fraction and not the protein precipitated at higher concentrations of sodium sulfate.

It is not possible to draw any more definite conclusions through a consideration of past work. Gibson and Collins (9) fractioned immune sera of various kinds from different animals and were unable to show that the agglutinins or antitoxins were associated exclusively with any particular fraction. They did find that a large proportion of a particular antibody was found either with the euglobulin fraction or with the pseudoglobulin fraction. These conclusions were reached after consideration of the literature in which immune bodies were held to be associated with definite fractions.

Hartley (10) has since held that the immune bodies against rinderpest were associated with the euglobulin fractions of blood serum. He also found an increase in the euglobulin fraction as the result of immunization. Homer (11) has

studied the fractionation of diphtheria antitoxin serum with sodium sulfate as well as with ammonium sulfate. With regard to the distribution of the antitoxin her work was similar to that of Gibson and Collins in that, by fractioning with different concentrations of ammonium or sodium sulfate, some of the antitoxin was found in the euglobulin-pseudoglobulin zone, but most of the antitoxin was found with the pseudoglobulin. It was further shown that "the percentage precipitation of the antitoxin with the proteins precipitated at various concentrations of sodium sulphate is a linear measure of the percentage precipitation of the antitoxin-bearing proteins." This relationship is undisturbed by heat denaturation of plasma adjusted to a definite hydrion concentration. There is a particular difference between our results and those of other investigators in that the proteins were precipitated at a relatively high dilution, a condition which should tend to minimize the effect of contamination by adsorption.

SUMMARY.

A comparison of the appearance of the agglutinins for *Bacillus abortus* in the blood of new-born calves with the first appearance of globulins in the same blood following the ingestion of colostrum indicates that the agglutinins are associated with the globulins. These observations are supported by the removal of the agglutinins from serum or colostrum with concentrations of sodium sulfate which precipitate globulins present in the blood of calves which have ingested colostrum, but which are not present at birth.

Neither the association of immune bodies with globulins nor the direct absorption of protein by new-born animals is a new fact. The evidence presented is of particular value, however, in associating the appearance of certain protein fractions in the blood of the new-born animal with the simultaneous absorption of agglutinins.

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THE SOURCE OF THE MICROORGANISMS IN THE LUNGS OF NORMAL ANIMALS.

BY F. S. JONES, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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Considerable discussion has arisen at various times relative to the sterility of the normal lung during life. Hildebrandt (1) claimed that inspired bacteria under normal conditions do not reach the lung but are deposited in the nasal passages. Babes (2) stated that the lungs of normal men and animals were sterile. The findings of Barthel (3) were similar. Beco (4) found organisms as far as the middle of the trachea in domestic animals but believed the lungs as a rule were sterile. On the other hand, the assertions that the lungs contain organisms are upheld by many. Neisser (5) in studying sections of the lungs of normal guinea pigs, rabbits, and mice encountered organisms in three instances. Dürck (6) found bacteria in the sixteen human lungs examined. Boni (7) succeeded in cultivating organisms from the lungs of fourteen swine. In eleven instances a pneumococcus of low virulence was obtained. From two of ten guinea pigs he obtained positive cultures. Nenninger (8) states that the lung cannot be considered as a germ-free organ since it is constantly invaded by organisms of the atmosphere and those from the upper respiratory passages. Thomson and Hewlett (9) calculated that at least 1,500 organisms were taken into the nasal passages per hour in London. They claim that the ciliary action of the lining epithelium soon rids the inspired air of microorganisms. They maintain that the lungs are sterile. An instance cited by them is an observation by Lister of a case of rib fracture in which the lung had been punctured and blood mixed with inspired air entered the pleural cavity. The blood did not undergo "decomposition." Lister pointed out that the particles filtered out before reaching the alveoli. In many instances the exact methods of cultivation have not been given by various observers.

Quensel (10) was able to show that the lungs were not bacteria-free. His material was obtained from the larger domestic animals immediately after slaughter at the abattoir. Pieces of normal lung were removed from the carcass as quickly as possible and transported to the laboratory in sterile glass containers. The surface of the tissue was then seared and bits removed with sterile instruments and crushed between sterile glass slides. The crushed material was used to inoculate tubes of agar and bouillon. Cultures were made from the lungs of

16 calves, 15 sheep, and 5 horses. From the calves, 23 of 62 tubes showed growth. Organisms were found in 19 of the 54 tubes from the sheep. The number of the positive tubes from the horses was relatively higher, 11 of 19 revealed organisms. Streptothrix, *B. subtilis*, and molds made up 64 per cent of the organisms cultivated. Streptothrix were most frequently obtained. Quensel in discussing his findings pointed to several possible sources of entrance. He suggested that the organisms may have entered the lungs with the passive flow of contaminated fluids (blood, stomach contents, etc.) during slaughter, or that they may have been carried downward during the last violent respirations. Air-borne contamination during the manipulations of the tissues was also considered as a source of error. He was inclined, however, to regard these organisms in most instances as legitimate inhabitants of the lungs. In sharp contrast to these findings is the statement that he found the lungs of guinea pigs free from bacteria.

Quensel was able in a few instances to cultivate streptothrix and other organisms from the bronchial lymph glands of the calf, horse, sheep, and pig.

It was known that the larger herbivora harbored organisms, but some doubt existed as to the presence of bacteria in the lungs of the laboratory animals. Their possible source and significance seemed of particular importance. With these points in view a series of observations and experiments was undertaken.

Cultural Studies from the Lungs of Animals.

The animals employed were apparently in good health. The smaller animals—such as rabbits, guinea pigs, mice, etc.—were chloroformed. It is customary to kill calves by stunning with a heavy blow on the head, the trachea is quickly clamped, and the large neck vessels severed. The same methods were employed in certain instances in the smaller animals but it was found that the method of killing did not materially alter the findings in the case of the rabbit or guinea pig.

The hair over the chest of the laboratory animals was shaved and the skin cleansed with 5 per cent phenol followed by alcohol. The skin was reflected and the underlying musculature seared. A triangular window of ribs and sternum was removed with sterile instruments. Small pieces cut from the borders, furthest removed from the trachea, of the various lobes were added to tubes of media. In calves large pieces from the borders of two lobes were removed with sterile instruments through incisions in the chest walls. From the

external edges of these small pieces were used for cultivations. Relatively simple media, such as slanted plain agar and agar to which a few drops of defibrinated horse blood had been added and veal infusion bouillon were employed. The bits of tissue were pushed

TABLE I.

Results of the Bacteriological Examinations of the Lungs of Normal Animals.

Animal.	No. examined.	No. of tubes inoculated.	No. of tubes showing growth.	Kinds of organisms.	
Calves.	9	74	63	Streptothrix,	45.
				B. subtilis,	6.
				Molds,	8.
				Cocci,	2.
				Mixed (*B. subtilis* and streptothrix),	2.
Rabbits.	7	50	42	Streptothrix,	5.
				B. subtilis,	20.
				Molds,	7.
				Cocci,	7.
				Mixed (*B. subtilis*, streptothrix, and mold),	3.
Guinea pigs.	12	72	54	Streptothrix,	39.
				B. subtilis,	10.
				Molds,	3.
				Cocci,	2.
White mice.	8	36	11	Streptothrix,	5.
				B. subtilis,	3.
				Cocci,	3.
" rats.	6	36	13	Streptothrix,	1.
				B. subtilis,	2.
				Cocci,	2.
				Molds,	8.

down over the surface of the slants into the condensation fluid. All tubes were sealed with sealing wax. Tubes were incubated for 14 days at 38°C.

The results have been recorded in Table I.

The results recorded in Table I are striking. Among the herbivorous animals the proportion of tubes showing growth is consistently high, varying from 75 per cent in the case of the guinea pig to 85 per cent in the instance of the calf. In not a single instance did all the tubes inoculated from the lung remain sterile. The proportion of positive tubes from the mouse and rat is much lower, 31 and 38 per cent respectively.

It is probable that two of the sources suggested by Quensel for the presence of organisms in the lungs have been ruled out. The relatively simple technique precludes contamination to any great degree through manipulation of the tissues. That fluids such as blood and regurgitated material cannot enter the clamped trachea is obvious. Regurgitation of stomach contents has not occurred in the smaller animals killed with chloroform.

The following facts tend to show that the organisms are within the lung. If tubes containing bits of lung are examined at frequent intervals, the filaments or tufts of streptothrix are frequently seen growing out of the borders of the tissue mass. This has been observed repeatedly on both the pleural and cut borders. The rapid growths of *Bacillus subtilis* and molds begin from the water of condensation and extend upward.

Quensel's third contention, however, that organisms may have gained access during the last deep respirations is doubtless true. Should this be admitted, then it must be assumed that at many of the deep inspirations during life organisms may reach the lungs.

A striking feature is the great similarity of the type of organisms encountered in the various species, particularly in the herbivorous animals. Streptothrix appeared 87 times, *Bacillus subtilis* 41, molds 25. Practically all the organisms met with are spore bearers. It is well known that they are particularly abundant in hay and straw. It seemed probable that they originated in the hay and straw and were taken into the respiratory tract. This would account for the larger proportion cultivated from the herbivorous animals and explain their small numbers in the mouse and rat.

EXPERIMENTAL

To establish this hypothesis a series of experiments was undertaken.

Experiment 1.—Full grown healthy rabbits were placed in sterile individual cages in a separate unit and fed the usual ration of oats, hay, and mangels. All food was washed thoroughly in running water and fed while moist. Newspapers were used for litter. All litter and uneaten food were removed daily. The control rabbits received the same ration unwashed and fed dry. After periods of 11 days to 2 weeks the animals were chloroformed and cultures prepared from the borders of the lungs. Bits of tissue the same size were used in cultivating from both groups of rabbits. The results are given in Table II.

TABLE II.

Influence of Moistened and Dry Food on the Number of Organisms Found in the Lung.

Animal.	No. examined.	No. of tubes inoculated.	No. of tubes showing growth.	Kinds of organisms.	
Experimental rabbits (fed washed hay and oats).	7	48	14 (29 per cent).	Streptothrix,	7.
				B. subtilis,	7.
Control rabbits (fed in usual manner).	6	44	40 (91 per cent).	Streptothrix,	11.
				B. subtilis,	22.
				Molds,	1.
				Mixed (streptothrix, *B. subtilis*, and molds),	6.

The results recorded in Table II are significant. The actual decrease in the number of tubes containing growth is striking. The inference that forms usually occurring in hay and straw may reach the lung is well founded. Similar experiments were tried on the guinea pig. A diminution always occurred in the number of tubes containing organisms but the results were never so striking.

By modifying the experiment somewhat the results obtained were very much the same.

Experiment 2.—Guinea pigs of the same age were divided into three lots. The cages and the litter were similar for each lot. All were kept in the same general atmosphere. One lot was fed on cabbage and oats, another lot was fed on grass and oats. The other controls received hay and oats with a little grass. After a

period of 1 month all the animals were killed and cultures prepared from the lungs. Since the results from the cabbage-fed and grass-fed animals were the same, they have been grouped together in Table III.

Much the same result was obtained in the case of the guinea pigs as in the rabbits under practically dust-free conditions. The diminution in the number of tubes showing growth from those kept in a reasonably clean atmosphere by withholding dry food matter is quite

TABLE III.

Results of Cultivations from the Lungs of Guinea Pigs Fed Grass and Hay.

Animal.	No. examined.	No. of tubes inoculated.	No. of tubes showing growth.	Kinds of organisms.	
Guinea pigs (grass or cabbage fed in place of hay).	7	38	10 (26 per cent).	Streptothrix,	8.
				B. subtilis,	1.
				Molds,	1.
Control guinea pigs (ration the same except hay was fed).	7	37	33 (89 per cent).	Streptothrix,	22.
				B. subtilis,	7.
				Molds,	4.

remarkable. The few organisms encountered in the experimental lots probably originated in the general atmosphere. In species thickly covered with hair there is no reason to suppose that spores may not exist for long periods. Methods to control this factor were not employed.

It is interesting to compare the results of the lung cultivations of the mice and rats recorded in Table I with those given in Tables II and III for rabbits and guinea pigs whose environments in general paralleled those of the rats and mice. The proportion of tubes showing growth is comparable. The rats and mice examined were kept in the same room with the other experimental animals. The same litter was used. The ration consisted of dog biscuit and whole corn. Hay or straw was not used in the cages. It seemed possible to account for the smaller number of organisms by the absence of materials heavily contaminated with spores. There was a possibility of some differences in anatomical structure of the upper respiratory tract which might not permit the free passage of many spores.

Experiment 3.—To test these points, twelve mice were divided into three groups. Each group was placed in a glass jar and received the same food. Lot 1 was given sawdust for litter and fed the usual ration of dog biscuit and corn. In the case of Lot 2 a mixture of finely cut straw was used for litter, the ration was the same as for Lot 1. With Lot 3 the litter consisted of finely cut newspaper which was changed daily. Both the corn and dog biscuit were sterilized in the autoclave for 20 minutes. After 8 days the mice were chloroformed and examined. The results are recorded in Table IV.

As a result of this experiment the fact that the flora of the lungs of the mouse can be influenced markedly by variations in the immediate

TABLE IV.

Proportion of Organisms Obtained from the Lungs of White Mice Kept under Ordinary, Dusty, and Dust-Free Conditions.

Lot 1. Ordinary conditions.	Lot 2. Straw as litter.	Lot 3. Dust-free conditions.
Mice, 4.	Mice, 4.	Mice, 4.
Tubes inoculated, 20.	Tubes inoculated 20.	Tubes inoculated, 20.
" showing growth, 9.	" showing growth, 16.	" showing growth, 1.
" remaining sterile, 11.	" remaining sterile, 4.	" remaining sterile, 19.
Tubes positive, *per cent*, 45.	Tubes positive, *per cent*, 80.	Tubes positive, *per cent*, 5.
Growth obtained from the lungs of all mice.	Growth obtained from the lungs of all mice.	No growth obtained from the lungs of three mice.

environment is brought out. Mice under ordinary conditions (Lot 1) show a moderate number of organisms within the lungs. The number can be increased until they approximate those found in the guinea pig or rabbit under normal conditions by subjecting them to the usual environment of the latter species. Evidently anatomical conditions are not responsible for the relatively few organisms encountered under usual methods of life. In Lot 3, in which the air was relatively free from dust and the spores of many forms, probably few organisms reached the lungs.

The experiments all tended to point to the immediate environment as the source of the organisms cultivated from the lungs. By withholding or supplying food and litter substances, such as hay and

straw, it has been possible to influence markedly the number of spore-bearing organisms cultivated. If it were possible to show that the streptothrix isolated from the lung resembled those found in the dust from hay, the chain of evidence would be complete. It has been stated that streptothrix are present in the greatest number of cultures. It was determined to compare the common lung strains with those obtained by exposing sterile plates to dust from hay and straw.

TABLE V.

Characters of the Streptothrix Obtained from the Lungs of Various Animals and Plates Exposed to the Dust of Hay and Straw.

Source and No. of strains.	Gram's stain.	Hemolysis in horse blood agar plate culture.	Milk.	Final hydrogen ion concentration in dextrose bouillon.	Gelatin.
				pH	
Lungs of guinea pigs, 26. " " rabbits, 4. " " calves, 6. " " mice, 5. Plates exposed to hay and straw, 5.	+	+	Coagulated, alkaline, curd peptonized.	7.6–8.6	Liquefied.
Lungs of guinea pigs, 3. " " calves, 4. " " mice, 4. Plates exposed to hay and straw, 2.	+	−	" "	7.6–8.6	"

All cultures were Gram-positive filaments with true branches. The surface growth always became chalky as the media dried and the characteristic fragmentation of the filaments with the production of arthrospores was observed. Most of the cultures had a characteristic penetrating musty odor. Their biochemic activities are given in Table V.

The general characters of the streptothrix from the lungs of the guinea pig, rabbit, and calf resemble those obtained from hay and

straw. All liquefy gelatin, coagulate milk, and peptonize the curd. Alkali is produced in dextrose broth and milk. Some hemolyze horse blood in agar plate cultures, others fail to do so. The predominating color on potato is yellow, yellowish white, or orange. The rapidity with which gelatin and casein are digested varies in different cultures. Unfortunately, these are the characters given for most of the streptothrix but radical departures from these types have not been met with so that one feels safe in considering these organisms of the same group.

Quensel was able to cultivate streptothrix and *Bacillus subtilis* from the bronchial lymph glands of various animals in a few instances.

TABLE VI.

Results of Inoculations from the Bronchial Lymph Nodes of Guinea Pigs.

No. of animals examined.	No. of tubes inoculated.	No. of tubes showing growth.	Kinds of organisms.	
11	63	42 (66⅔ per cent).	Streptothrix,	28.
			B. subtilis,	11.
			Cocci,	1.
			Short rods,	1.
			Pleomorphic rods,	1.

To augment his findings and perhaps throw some light on their significance in the lymph glands a series of cultivations was made. The bronchial lymph glands in the guinea pig are easily visible and small enough that all of them may be used for culture material. A relatively simple technique has been devised with which the liability of contamination has been reduced to a minimum. The results are given in Table VI.

66⅔ per cent of the tubes showed growth. In no instance were all the bronchial lymph nodes from any guinea pig sterile. The organisms encountered are similar to those observed in the lung and probably were forms that had been taken into the alveoli and smaller bronchioles and transported to the draining lymph glands.

The mechanism of phagocytosis of bacteria and other foreign bodies within the lung has been studied by many. The more plausible view of Haythorn (11), Permar (12), and others is that dust particles

and certain bacteria are taken up by endothelial cells from the blood vessels. These cells make their way into the air space, and pass out through the walls entering the lymph spaces and ultimately reach the lymph nodes. This seems to offer a satisfactory explanation for the appearance of certain organisms in the lymph nodes. It has been possible to study certain phases of this mechanism. If spores of streptothrix and finely divided particles of carmine, or the dye and *Bacillus subtilis* are suspended in salt solution and injected intratracheally into guinea pigs, after 1 or 2 hours cells which have taken up both materials can be demonstrated in wet preparations or in stained films from the lungs. Many of these cells in size and general morphology conform to the macrophage. Streptothrix in large numbers may be recovered from the bronchial lymph glands 12 to 18 hours after their injection into the trachea.

Quensel was able to isolate organisms in but a small number of instances from the lymph glands of horses, calves, sheep, and swine. The great differences which have been found in the guinea pig may be explained on the proportionate bulk of the tissue examined. With the larger animals comparatively little material was actually cultured. All the visible bronchial lymphoid tissue was used from the guinea pig. In the large animals the distances to the draining lymph glands are greater and a longer time interval is afforded for the destruction of phagocytosed organisms before the phagocytes reach the draining lymph nodes.

DISCUSSION.

From the preceding experiments it is obvious that the lung is readily invaded by air-borne organisms. It hardly seems possible that organisms the size of streptothrix, the type most frequently found, the molds, or even bacteria of the *subtilis* group are capable of vegetating and multiplying within either the smaller bronchi or alveoli. Even moderate multiplication would doubtless lead to serious mechanical disturbance. It seems more reasonable to assume that theoer sps of these various types which abound in dry vegetable matter are taken into the respiratory tract with each inspiration. During deeper breathing a number must reach the smaller air passages and alveoli. The statement that they reach the small bronchioles and alveoli

appears warranted when it is considered that only borders furthest removed from the principal bronchi were used for cultivation. Tufts of streptothrix were observed to grow through the pleura as well as the cut surfaces, evidently in these instances they were closer to the pleura and grew from alveoli.

The organisms are non-pathogenic when injected subcutaneously. The spores then are comparatively inert and are taken care of by the same mechanism that functions in the case of coal dust and other inert matter. This seems to explain their presence in the bronchial lymph nodes. It may be argued that the cells responsible for phagocytosis do not in all instances destroy the injected spores promptly. Some certainly reach the lymph glands in a viable condition and are doubtless ultimately destroyed there. Rous and Jones (13) were able to show experimentally that living phagocytes were enabled to protect injected organisms from the action of destructive substances in the surrounding fluid. Briscoe (14) in his experiments on phagocytosis in the lungs following intratracheal injection of bacteria and foreign blood corpuscles showed that after 1 or 2 hours the non-pathogenic organisms and red cells had been taken up by mononuclear cells. The polymorphonuclear leucocytes did not appear to any great extent until later. Briscoe's interpretation of the origin of these mononuclear cells differs from that of Haythorn and Permar.

It has been possible with calves to augment the reported findings. The most frequent organisms encountered in the normal nasal passages of calves have been streptothrix and molds. It has been possible to cultivate similar organisms from the trachea. The experiments in which mixtures of carmine and spores and carmine and vegetative forms were injected into the trachea and in many instances taken up by large cells with single nuclei are additional evidence for the hypothesis. The recovery of streptothrix from the bronchial lymph glands in large numbers 12 hours after the injection into the trachea is also significant.

Cocci of various kinds have been cultivated in a relatively small proportion of animals. The assumption has been to regard them as originating in the upper respiratory tract.

There is no reason to assume a different route of entrance in the case of usual types of animal pathogens. The cilia and other factors

are probably no more capable of keeping out the small vegetative pathogens than the spore forms of the non-pathogenic varieties.

The observations have some practical bearing for those interested in the study of respiratory disease, especially in species like the rabbit and guinea pig. By withholding spore-bearing subtances, such as hay and straw, it is possible to cut down the number of contaminating organisms. This is of considerable value where small, oftentimes indistinct foci of consolidation are met with.

SUMMARY.

It has been possible to show that the lungs of such animals as the calf, rabbit, guinea pig, white rat, and white mouse are readily invaded by organisms. The most frequent types observed in cultures from the border of the lungs have been streptothrix, molds, and bacteria of the *Bacillus subtilis* group. These forms originate in certain dry foodstuffs (hay and straw). By withholding or moistening these materials it has been possible to diminish the number of organisms in the lungs. When these materials have been supplied to mice whose lungs under usual conditions contain only a few organisms, the number of positive cultures increases and is comparable with those of the larger animals. The bronchial lymph glands of all guinea pigs examined developed, in 66⅔ per cent of the tubes, organisms similar to those obtained from the lungs.

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IPECAC IN THE TREATMENT OF BLACKHEAD IN TURKEYS.

BY H. W. GRAYBILL.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey.)

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The spring of 1921 Wegeforth and Wegeforth (1) reported results on the treatment of infectious enterohepatitis or blackhead in turkeys. During a period of six seasons they experienced the usual difficulties of those undertaking the rearing of this bird. In September, 1919, after their flock of that year had been decimated and when the outbreak was still in progress, there remained 52 young birds out of a flock of 209 hatched. About that time the early researches of Dr. Theobald Smith on the etiology of the disease came to their attention. The presence of Amoeba meleagridis in the lesions suggested as a remedy ipecac, a drug which has proved of value in the treatment of amoebic dysentery in man. They pursued the plan of isolating birds as soon as symptoms developed and placing them under treatment. During the first three days 10 drop doses of fluid extract of ipecac were administered per os three times a day, then for three days two 10 drop doses, and for three days longer one 10 drop dose. Of the 52 birds remaining 32 were attacked by the disease. Of these 29 recovered, making a mortality of about 9 per cent. Of five old birds that developed the disease, three recovered and two died.

In two additional experiments, the authors obtained results which led them to believe that the disease was prevented by the use of fluid extract of ipecac and by powdered ipecac.

In view of these experiments it seemed desirable that further tests with ipecac should be made.

Experiments with Ipecac.

During the summer of 1921 a spontaneous outbreak of blackhead supplied a number of cases which were used to test out the value of

fluid extract of ipecac as a remedy. The flock was running in a small enclosure and as soon as a bird showed symptoms it was transferred to a nearby pen provided for sick turkeys and at once placed under treatment. The age of the birds when they became ill ranged from twenty-nine to sixty-two days. The doses were usually diluted with a somewhat greater quantity of water. The course of the treatment was adjusted as far as possible to the condition of the bird. Sometimes treatment was omitted for a time because the bird was considered too sick, at other times treatment appeared unnecessary on account of a marked improvement in condition.

In the accompanying table the dosage and results of treatment are given. Nineteen turkeys were treated. Of these, 9 died of blackhead and the rest recovered making a mortality of nearly 50 per cent.

At the time the first turkeys became available for treatment no tests for determining dosage had been made. A few tests were then made on normal turkeys, and later the two controls (no. 20 and no. 21) shown in the table were maintained.

The tests on normal turkeys were on birds twenty-nine to thirty-four days old.

The results were as follows: ten drops a day for two days in 5 drop doses did not prove toxic. Ten drop doses a day for four days proved slightly toxic. Two 10 drop doses for one day and one on the following day proved somewhat toxic. Two 8 drop doses for one day and one 8 drop dose the following day proved somewhat toxic. Two 10 drop doses for one day proved slightly toxic.

The determination of toxicity was based on the presence of symptoms of illness, such as not being normally active, or drooping.

From these results it will be noted by referring to the table that some of the birds received toxic doses. It will therefore be well to consider individually those turkeys that died.

Turkey 1 on the fourth day after treatment began appeared normal indicating that there was no important toxic effects at that time. On the fifth and sixth days it was not quite normally active. Treatment was then discontinued. It grew worse and died five days later.

Turkey 4 had improved on the fourth day so that it was normally active. On the fifth and sixth days it was not quite normally active. Then followed a period of six days when treatment was discontinued.

No. of turkey.	Age.	Dates of treatment and number of drops given.*																														Remarks.
		July.																										August.				
		1	2	3	4	5	6	11	12	13	14	15	16	17	18	19	20	21	22	23	25	26	27	28	29	30	1	2	3	4	
	days																														
1	36	10		10	10	10	10																								Died July 11.
		10																													
2	29	5	5	10	10																										Recovered.
3	36	5	5	10			10																								Recovered.
		5	5																												
4	36	10		10	10	10	10			10																					Died July 14.
		10																													
5	36	10		10	10	10	10			10	10		5	5	5	5	5	7													Died July 22.
		10																													
6	36	5	5	10	10	10	10			10	10			5	5	5	5	7	5												Recovered.
		5	5																												
7	39							10	10																						Died July 13.
8	40					10	10			10	10																				Died July 16.
9	39							10	10	10	10		5	5	5	5	5	7													Died July 22.
10	46							10	10	10	10																				Died July 15.
11	41									10	10		5	5	5	5	5	7	5												Recovered.
12	41									10	10		5	5	5	5	5	7	5												Recovered.
13	41									10	10		5	5	5	5	5	7	5												Recovered.
14	44												5	5	5	5	5	7	5	6	6	6	6								Died July 28.
15	48																10		10	6	6	6	6	6	6	6	6				Died August 7.
16	53																				6	6	6	6	6	6	6	6	6	6	Recovered.
17	53																				6	6	6	6	6	6	6	6	6	6	Recovered.
18	53																				6	6	6	6	6	6	6	6	6	6	Recovered.
19	62																						10	10	6	6	6	6	6	6	Recovered.
20	43											6	5	5	5	5	5	7	5	6	6	6	6	6	6	6					No toxic effects.
(Control)																															
21	50											6	5	5	5	5	5	7	5	6	6	6	6	6	6	6					No toxic effects.
(Control)																															

*Two figures on same date indicate two doses were given.

It improved during the first half of this period and appeared normal. Then it was not normally active again and died, following another treatment, at the end of fourteen days.

Turkey 5 likewise showed improvement and appeared normal by the fourth day. On the fifth and sixth days it was not quite normal. During an interval of six days when treatment was suspended it first showed improvement to normal and then showed symptoms again. On the thirteenth day it was decidedly sick and treatment was begun again, the dose being decreased after two days. The last course of treatment could hardly be regarded as toxic. It died twenty-two days after treatment was begun.

Turkey 6 received practically the same course of treatment and recovered.

The dosage for *turkeys 7 and 8* cannot be regarded as toxic according to tests made. Treatment of no. 7 was suspended on account of death, and of no. 8 was discontinued for a time on account of its improved condition.

Turkey 9 showed improvement on the sixth to eighth day after treatment began, then became worse, and died four days later, one day after last treatment.

Turkey 10 received 10 drops on four days. In the tests made on normal turkeys, four doses were found only slightly toxic in a considerably younger turkey.

In the two remaining turkeys that died (*no. 14* and *no. 15*) the treatment was sufficiently controlled by turkeys no. 20 and no. 21.

From the above survey of the treatment and condition of the birds, it does not seem probable that the treatment could have been injurious to any considerable degree.

The lesions found in the birds that died presented the usual appearance found in untreated birds. In six the liver and both ceca were involved, in two the liver and one cecum, and in the remaining one the liver was normal but both ceca were involved. The case of turkey 9 was complicated by aspergillosis.

DISCUSSION AND CONCLUSIONS.

The absence of a control group of untreated diseased turkeys renders it impossible to indicate how the degree of mortality in the treated

turkeys stands to that which would have occurred in the absence of treatment. It should be stated that the mortality was less than usually occurs in young birds. However, our results do not confirm those of Wegeforth and Wegeforth who used turkeys considerably older. It is well known that the resistance of birds to manifest disease presumably as a result of infection increases with age and it is possible that such resistance played an important part in the results obtained by the authors referred to. A mortality of approximately 50 per cent, such as we obtained, indicates that if ipecac has any value in the treatment of blackhead it cannot in all probability be very great.

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TRANSPARENT MILK AS A BACTERIOLOGICAL MEDIUM.

BY J. HOWARD BROWN AND PAUL E. HOWE.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey.)

(Received for publication, February 10, 1922.)

The addition to milk of an oxalate or a citrate, in the form, for example, of a sodium salt, will cause the opacity of milk to disappear and give a solution which is opalescent in thick layers but almost clear in thin layers.[1] A similar result may be obtained with sodium sulphate when added in relatively larger amounts. This change is particularly evident when the milk is diluted slightly. Although we have not found the phenomenon described in the literature it may have been observed by those who for infant feeding have added sodium citrate to milk to prevent rennet coagulation. The general nature of the reaction has been known and the procedure has been used to prevent the coagulation of blood. Arthus (1902) showed that sodium citrate in the proportion of 2 to 3 parts per 1000 would prevent the coagulation of milk by rennin. Bosworth and Van Slyke (1914) in a study of "why sodium citrate prevents curdling of milk by rennin" found that there was an increase in the amount of soluble calcium which would pass through a Chamberland filter with increasing quantities of sodium citrate until approximately 1 gram of the hydrated salt had been added to 100 cc. of milk. Rennin coagulation was prevented when 0.4 gram of the citrate had been added to 100 cc. of milk. The failure to coagulate in the presence of citrates was ascribed to the formation of calcium citrate or sodium-calcium citrate.

The loss of opacity is apparently due to the removal of the calcium combined with casein or globulins in milk. The addition of oxalate or sulphate results in the formation of insoluble calcium oxalate or sulphate which may be removed by centrifugation. By the addition

[1] Howe, Paul E., unpublished work.

of sodium citrate, however, the calcium of milk is not precipitated (Arthus, 1902), but remains in solution in a non-effective form in so far as its ability to combine with casein or to react with the para-casein formed as a result of the action of rennin are concerned. We have not found an adequate explanation of the nature of the calcium-citrate combination. That it is in solution is indicated by the work of Arthus and of Bosworth and Van Slyke. We have evidence of this in the fact that during each sterilization there is formed a heavy precipitate of a calcium salt which we assume to be calcium citrate. It has the property of calcium citrate of being precipitated when heated and redissolving upon cooling. On the other hand, the calcium in solution in the clarified milk must be sufficient to form the insoluble para-casein compound for there is enough calcium in milk to do so, and in the preparation of transparent citrated milk none need be removed. Sabbatani (1902) states that the effect of the citrate in blood is due to a reduction in the number of calcium *ions* in solution. He also shows that the ratio of citrate to calcium for the prevention of coagulation is three to one. Arthus holds that there is a specific effect of the citrate ion which inhibits flocculation, in addition to any changes which may take place in solubility.

In the preparation of transparent milk as a bacteriological medium we have diluted 1 part of skim milk with 2 parts of distilled water and then added 0.4 per cent of sodium citrate. After standing for about an hour the clarified milk may be filtered through paper though this is hardly necessary if the mixture is allowed to stand sufficiently long. To avoid caramelization of the milk sugar during sterilization the reaction of the medium is adjusted to about pH 6.8. The medium is then tubed and sterilized fractionally. During each steaming in the Arnold sterilizer a heavy precipitate is thrown down but redissolves as the medium cools. There finally results an almost water-clear medium without any precipitate whatever. Oxalated milk may be prepared in the same manner except that the fine precipitate of calcium oxalate should be removed by centrifugation. For most purposes the citrated milk appears to be the more satisfactory medium, since the citrate gives changes which correspond with those which take place in milk without further treatment. Citrates are a normal constituent of milk.

We have used these media for the cultivation of a number of organisms—streptococci, anaerobes, and members of the colon-typhoid group. A few organisms grow better in the citrated milk than in oxalated milk, though the latter is useful for special purposes because of the removal from it of the calcium. The citrated milk shows all the cultural reactions which may be observed in untreated milk of the same dilution. Some organisms, notably the paratyphoids, produce a reaction not to be observed in untreated milk. These produce in citrated milk a milky translucence or opacity which we attribute to the decomposition of the citrate with liberation of calcium in the ionized state, an appearance which may be produced artificially by the addition of a small amount of calcium chloride to the sterile medium. This reaction is not produced by cultures in oxalated milk from which the calcium has been removed.

The cultural reactions which we have observed in transparent citrated milk may be summarized as follows:

I. Neither acid production nor digestion of casein. Reaction alkaline or neutral.
   a. The medium remains clear except for the clouding due to visible growth. The addition of a few drops of $CaCl_2$ solution causes it to become milky. Example: Bact. typhosum. Bact. alkaligenes.
   b. The medium becomes milky, probably due to a release of ionized calcium. Example: Bact. paratyphosum, Bact. cholerae-suis.

II. Acid production.
   a. A small amount of acid may do nothing more than change the color of the indicator which may be added to the medium. Example: Strep. pyogenes.
   b. A larger amount of acid may produce translucence. Often observed as a transitory reaction. Example: Bact. cloacae.
   c. Large amounts of acid produce coagulation or precipitation of casein. Example: Strep. lacticus, Lactobacillus bulgaricus, Bact. coli, Clostridium welchii.

III. Rennin production.
   a. Without release of calcium from citrate should give precipitate when calcium chloride is added unless the casein has been digested.
   b. With release of calcium from citrate should produce precipitate or coagulum.

IV. Casein digestion.
   a. Should give diminished or negative precipitation upon addition of acetic acid. Example: Proteus vulgaris.

Various combinations of the above reactions may be observed and there are doubtless other reactions which are not mentioned above, for instance, with alkali production we have sometimes observed a thickening of the medium into a transparent jelly. This reaction was produced by Bact. alkaligenes.

SUMMARY.

Milk may be transformed into a transparent culture medium by the addition of small amounts of various salts. Sodium citrate seems to be the most suitable for this purpose.

In such a medium there may be observed not only the ordinary reactions of various bacteria in milk but also some others not observed in untreated milk.

The principal advantages of the transparent milk as a medium reside in the greater visibility of changes which occur in it. Indicators are much more easily seen in it than in opaque milk. As long as the acidity remains below pH 5.5 colorimetric hydrogen ion determinations are easily made. Clouding and the formation of sediment due to bacterial growth may be observed as in bouillon when no visible change whatever is produced in ordinary opaque milk.

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